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Barnett, J. W. & Robinson, F. A. (1942) *Biochem J* 36, 364

Culbertson, C. C. & Thomas, B. H. (1933) *Rep Agric Res Iowa St Coll.* 32

Doisy, E. A., Somogyi, M. & Shaffer, P. A. (1923) *J biol Chem* 55, Proc xxxi

Fairley, N. H. (1938) *Nature, Lond.*, 142, 1156

Hennessy, D. J. (1941) *Industr Engng Chem (Anal ed.)*, 13, 216

King, H. (1941) *J chem Soc* p 338

Osborne, T. B. & Mendel, L. B. (1914a) *J biol. Chem* 17, 325

Osborne, T. B. & Mendel, L. B. (1914b) *J biol Chem* 18, 1

Osborne, T. B. & Mendel, L. B. (1916) *Biochem J* 10, 534.

Osborne, T. B., Mendel, L. B. & Ferry, E. L. (1919) *J biol Chem* 37, 233

Starling, E. H. (1915) *Principles of Human Physiology*, 2nd ed. London Churchill

Statistical Treatment of Data In general the publication is not necessary of all the individual results of a number of similar experiments. A statement of the number of individual results, their mean value, the standard error of the mean value, and the extreme range (highest and lowest values in the series) is usually sufficient.

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Microfungi should be designated as in Ainsworth & Bisby's (1945) *A Dictionary of the Fungi*, 2nd ed (Kew Imperial Mycological Institute).

Bacteria The Editorial Board prefers that the nomenclature of Bergey's *Manual of Determinative Bacteriology* (1948), 6th ed. (London: Baillière, Tindall & Cox) should be followed. Where authors wish, for good reasons, to use a name other than that in Bergey's *Manual*, the name as in Bergey's *Manual* should be inserted in brackets at the first full citation, thus *Chromobacterium prodigiosum* (*Serratia marcescens*).

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CORRIGENDA

The Fate of Certain Organic Acids and Amides in the Rabbit

3 Hydrolysis of Amides by Enzymes *in vitro*

By H G Bray, S P James, B E Ryman and W V Thorpe

Volume 42 (1948), No 2, p 277, Fig. 3

for 0 0174*m read* 0 0087*m*

for 0 0087*m read* 0 0174*m*

The Fate of Certain Organic Acids and Amides in the Rabbit

4 The Aminobenzoic Acids and their Amides

By H G Bray, H J Lake, F C Neale, W V Thorpe and P B Wood

Volume 42 (1948), No 3, p 443, col 1

Third reference *for* Marshall, J K Jr
read Marshall, E K Jr

Metabolism of Fluorane in the Rabbit

By W J P Neish

Volumes 43 (1948), No 4, p 534, col 1

line 5 *insert* the quinones of *after* that

line 7 *for* hydrocarbons *read* compounds

p 536, col 2

line 5 of 'Summary' *for* hydrocarbon *read* compound

The Inhibition of β Glucuronidase by Saccharic Acid and
the Role of the Enzyme in Glucuronic Synthesis

By M C Karunaratnam and G A Levvy

Volume 44 (1949), No 5, p 603, col 2

line 21 *for* methylglucuronide *read* menthylglucuronide

The Fate of Certain Organic Acids and Amides in the Rabbit

7 An Amide of Rabbit Liver

By H G Bray, S P James, I M Raffan, B E Ryman and W V Thorpe

Volume 44 (1949), No. 5, p 621, Table 3

Heading to col 4 *for* glutamine *read* glutamine*

p 624, col 1

line 2 from end of text *for* acetylglycine *read* glycyglycine

Volume 45 (1949), No 1, Index of authors

for Dale, W M p 106 *read* Dale, W M p 93

Preparation of the Antibiotic Nisin

By N J Berridge

Volume 45 (1949), No 4, p 486

for Received 20 May 1940 *read* Received 20 May 1949

The Turnover of Radioactive Phosphate Injected
into the Subarachnoid Space of the Brain of the Rat

By O Lindberg and L Ernster

Volume 46 (1950), No 1, p 44, col 1

line 14 *for* 105 *read* 10⁵

p 46, Table 4, last col

for 29 5 *read* 58 4 *for* 58 4 *read* 29 5

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 Entries marked with a section mark (§) refer to contributions made to Symposia
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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The 270th Meeting of the Biochemical Society was held at the London School of Hygiene and Tropical Medicine, Keppel Street, London, W O 1, on Saturday, 30 October 1948 at 11 a.m., and took the form of a Symposium on 'Partition Chromatography and its Application to Biochemical Problems'

COMMUNICATIONS

Inductory and Theoretical Aspects By A J P MARTIN (*Lister Institute of Preventive Medicine, London, S W 1*)

1 Purification processes may be classified into (a) separation of already preexisting phases, e.g. filtration, (b) distribution between phases followed by separation of the phases by processes described under (a), (c) establishment of a concentration gradient or difference by some diffusional process, followed by separation of regions of high and low concentration of some particular component

2 Purification processes belonging to any of the above classes may be applied by means of appropriate apparatus in a counter current manner. When the enrichment obtainable in a single simple operation is small, useful separation, i.e. a large factor of enrichment as well as a high yield, may thus be obtained. The chromatogram utilizing (a) filtration and (b) distribution between two phases is perhaps the most elegant example of the counter current process.

3 In the separation of two components by distribution between phases the enrichment factor is a measure of the difference between the amounts

of energy required to move the two components from one phase to the other. As a first approximation a given group in any molecule may be assumed to require a characteristic amount of energy on transference between a given pair of phases. Analogies can be drawn for partition between liquids, adsorption on solids and possibly crystallization, ionic, hydrogen bonding and Van der Waals forces, etc., playing easily recognizable roles. It is in many cases difficult and even meaningless to draw a sharp distinction between adsorption and partition chromatograms.

4 As in adsorption chromatograms, in partition chromatograms elution development, front analysis or displacement development can be used. Displacement development has advantages in the mass of material that can be handled. So far, change of pH has been the effective agent in causing displacement, but other possibilities exist, e.g. association with hydrogen bonding groups.

Applications of Partition Chromatography to Studies of Protein Structure By F SANGER (*School of Biochemistry, University of Cambridge*)

The most important problem before the protein chemist to-day is the elucidation of the order in which amino acids are arranged in a protein molecule, and this can probably only be solved by investigating partial degradation products of proteins. The fine resolving power of partition chromatographic methods is making possible the study of these extremely complex mixtures of amino acids and peptides.

The method of paper chromatography facilitates the rapid identification of any of the naturally occurring amino acids in a mixture, and has been applied to many problems of protein structure. The determination of the monoamino monocarboxylic acids by fractionation of their acetyl derivatives on silica gel columns was the first accurate method for estimating this group. Amino acids can also be estimated with considerable

accuracy after direct fractionation on starch columns or by fractionation of their radioactive *p*-iodophenyl sulphonyl derivatives on paper chromatograms.

Paper chromatography has now been extended to the fractionation of simpler peptides and the determination of their structure. Thus the order of the residues in 'gramicidin S' has been shown to be valyl ornithyl leucyl phenylalanyl prolyl, and a large number of dipeptides have been detected in partial hydrolysates of wool.

A method was worked out for the detection and estimation of the free amino groups of proteins and peptides using 2,4,6-trinitrofluorobenzene. This method has also been extended to the separation of peptides occupying terminal positions in the protein chains.

Applications to Studies of Amino-acid and Protein Metabolism By C E DENT (University College Hospital Medical School, London, W C 1)

So far, only paper chromatographic methods have been reported. These appear to be of great value for qualitative and rough quantitative analysis of amino acid containing fluids, especially of the free amino acids in tissue fluids. Several new amino acids exist in these fractions and are widely distributed in nature.

The most useful technical advance has been the invention of a desalting device by Consden, Gordon & Martin (1944). This enables much larger volumes of solution to be analyzed on the paper, so that most amino acids can now be readily detected at a concentration of only 1 $\mu\text{g/ml}$. For purposes of reference a map has been prepared showing the positions occupied on phenol 'collidine' two dimensional chromatograms by some 60 amino acids and other ninhydrin reacting compounds.

Changes in many individual amino acid concentrations in blood, urine and other fluids can be

readily observed simultaneously on paper chromatograms. This wide coverage of many of the possible variables is essential when the metabolism of single amino acids is being followed owing to the remarkable effect that one amino acid may have on the amounts of the others present. When the digestion and adsorption of proteins is being studied, changes in blood amino acids can provide data as to the likely mechanisms involved, and as to the availability of certain amino acids in the protein, and whether their rate of liberation by the gut enzymes is simultaneous or otherwise.

Five human diseases exist, the nature of which suggests some connexion with a gross error of amino acid or of protein metabolism. These are acute yellow atrophy of the liver, the Fanconi syndrome, cystinuria, phenylketonuria, and multiple myelomatosis. Preliminary work on these diseases was reported.

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Application to Carbohydrate Studies By S. M. PARTRIDGE (Low Temperature Research Station, University of Cambridge)

The introduction of partition chromatography by Martin & Synge (1941) and its further development by Consden, Gordon & Martin (1944) to the separation of free amino acids on filter paper chromatograms opened a field for the application of similar principles to the chromatographic separation of a fairly wide range of substances other than the amino acids. Thus simple sugars and oligosaccharides of low molecular weight were separated on filter paper by irrigation with solvents such as phenol-water, collidine water or butanol acetic acid water. The reaction of the sugars with a solution of silver nitrate in ammonia or a solution of naphthoresorcinol in trichloroacetic acid was used as a means of revealing their position on the chromatograms. When inorganic salts were present in the test solution, these were first removed by treatment with ion-exchange reagents (Partridge, 1946, 1948a, b).

Interference due to electrolytes was studied by

R. G. Westall (1948), while Jermyn & Isherwood (1948) investigated a wide range of solvent mixtures and obtained a marked improvement in the separations by irrigating paper chromatograms with ethyl acetate acetic acid water and ethyl acetate pyridine water mixtures. The selectivity of the method was also improved by Forsyth (1948), who examined the colours produced by the sugar spots on heating with various phenolic reagents in the presence of hydrochloric acid.

Quantitative applications were explored by Hawthorne (1947) and by Flood, Hirst & Jones (1947). Bell (1944) carried out the quantitative separation of a number of methylated sugars on a column of silica gel, and Brown, Hirst, Hough, Jones & Wadman (1948) applied the filter paper technique to the identification of methylated sugars in the complicated mixtures resulting from the hydrolysis of methylated polysaccharides.

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the presence of bromine water. The use of vanadic acid was suggested by an observation of Pozzi Escot (1946). The yield of pentabromoacetone is practically quantitative, large excesses of oxidizing agents are harmless, the presence of many natural substrates in excess does not interfere and the specificity of the reaction is increased, as, for example, hydroxy butyric acid gives no colour.

As for the colorimetric estimation of the penta

bromoacetone formed a number of recently described modifications were tried, but it is held that the sodium sulphide method (Pucher, Sherman & Vickery, 1936) is still superior to any other from the point of view of speed, simplicity, specificity, and sensitivity. No stabilizing agent is required if the petroleum ether used for extraction of pentabromoacetone has been properly purified and if the coloured extract is protected from strong light.

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The Hexokinase Reaction as an Indicator of Hormonal Levels in Human Blood By H. WEIL-MATHERBE (*Runwell Hospital, nr. Wickford, Essex*)

Evidence is mounting to show that the phosphorylation of glucose catalyzed by hexokinase is under direct hormonal control, being inhibited by pituitary and adrenocortical hormones and reactivated by insulin.

It has been found that the activity of rat brain hexokinase is greatly influenced by the addition of certain samples of plasma or corpuscle haemolysate of human blood, withdrawn from the antecubital vein of (1) 'normal' subjects, (2) diabetics, (3) patients during and after insulin hypoglycaemia.

Effects of plasma. Addition of plasma from normal fasting subjects to the hexokinase system is usually without significant effect, but after food intake activation may increasingly develop. On the small sample of diabetic patients available, the opposite effect was observed. A post absorptive inhibition occurred which, after insulin medication, was absent or replaced by activation. During insulin hypoglycaemia three types of response were observed: (1) absence of marked activation or inhibition, (2) prevalence of inhibition (more frequent in men), (3) strong activation (more common in women).

These effects may be explained by the assumption that inhibiting and activating factors are present in plasma in varying relative amounts.

Effects of corpuscle haemolysates. These usually show a strong activating effect already in specimens from fasting subjects. It is particularly noteworthy that this activation occurs also with 'diabetic' haemolysates. Haemolysates from hypoglycaemic patients may show an inhibition effect, especially when there is inhibition by the plasma. Otherwise there is little correlation between the effects of haemolysates and plasma.

The effects described cannot be accounted for either by unspecific protein effects or by the presence of hexokinase or ATP in the samples.

Commercial insulin ('A.B.') added *in vitro* (0.5 unit/ml) was found to have no significant effect on rat brain hexokinase activity, either in presence or absence of blood samples. In particular it does not reverse the inhibiting effects of samples of diabetic or hypoglycaemic plasma.

The Metabolism of N-Acetylglucosamine by Streptococci By H. J. ROGERS (*Biological Research Department, Dental School and Hospital, Leeds University*)

Despite the importance of amino sugars as constituents of a wide variety of polysaccharides, such as chondroitin sulphate, hyaluronic acid, chitin and those present in saliva or other mucins, little is known about their metabolism by either animals or bacteria. Lutwak Mann (1941) examined in some detail the breakdown of glucosamine by animal tissues and by a few strains of bacteria concluding

that kidney, testis and the bacteria contain powerful enzyme systems which oxidize and deaminate the amino sugar. Both aspects of the process were found to be inhibited by monoiodoacetate, fluoride, toluene and merthiolate, whereas cyanide was much more effective against oxidation than deamination. An acid substance was formed during oxidation which was neither lactic nor acetic acids.

In the present work the streptococci have been selected for study because some are capable of synthesizing comparatively large amounts of hexosamine containing polysaccharides, whilst others can break down such macromolecules and utilize the liberated hexosamines. Thus it seemed likely that these organisms would have an active amino sugar metabolism.

The end-products formed from *N* acetylglucosamine by ten different strains of *Streptococcus haemolyticus* group A and group C and seven of *Streptococcus viridans* isolated from the mouth have been examined. The results show that 1 mole of *N* acetylglucosamine is broken down to give 1 mole of ammonia, 1 equivalent of volatile acid and, from 1.2 to 1.7 moles of a substance which possesses the properties of lactic acid. Washed suspensions of the organisms were used and the reactions conducted

at 37.5° and pH 7.2. The yields of lactic acid from the amino sugar are closely similar to those obtained from glucose. The rates of deamination, lactic acid formation and the utilization of *N*-acetylglucosamine are closely similar and there is no evidence of any lag in deamination. Lactic acid is formed from glucose twice to twenty times faster than from *N* acetylglucosamine. Concentrations of from 0.2 to 0.05M glucose partially inhibit deamination of the amino sugar. Deamination, utilization and lactic acid formation are all partially inhibited by fluoride, citrate, capryl alcohol, iodoacetate, bisulphite, dimedone and toluene. There is a suggestion that deamination is rather more easily inhibited than utilization by some of the reagents. The results in general suggest that the pyranose ring structure is possibly broken before deamination occurs.

Quantitative Estimation of Threonine and Phenylalanine Content of the Polymyxins By

EILEEN I. SHORT (Wellcome Research Laboratories, Beckenham, Kent)

The polymyxins are polypeptide antibiotics derived from various strains of *Bacillus polymyxa* (Brownlee & Jones, 1948). All have threonine as a constituent amino-acid and two of them, polymyxin 'B' and 'C', contain phenylalanine (Jones, 1948).

Available microchemical methods have been applied to the estimation of these two amino acids in hydrolysates of the antibiotics. Threonine has been estimated by Winnick's method (1942) using Conway diffusion cells.

Phenylalanine has been estimated by a modification of Kapeller-Adler's method (1932). Preliminary hydrolysis is not necessary since heating with the nitration mixture of potassium nitrate and concentrated sulphuric acid causes simultaneous hydrolysis and nitration. The nitro compound is then converted to the purple salt of di-*o*-dinitro benzoic acid with hydroxylamine hydrochloride and am-

monium sulphate followed by sodium hydroxide (Albanese, 1944).

The amino-acid content is directly related to antibiotic activity and the methods can therefore be applied to the estimation of the polymyxins.

The purest sample of polymyxin 'B' so far obtained (10,600 u/mg) contains 13.2% phenylalanine and 14.92% threonine. These values suggest that the amino acids are present in the polypeptide molecule in the ratio of two molecules of phenylalanine to three of threonine and that the minimum molecular weight of polymyxin 'B' is approximately 2500.

The purest sample of polymyxin 'E' examined (8850 u/mg) has a threonine content of 14.36%. The molecular weight corresponding to this figure would be of the order of (835)_n.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

Partition Chromatography of Organic Acids, Purines and Pyrimidines By S R ELSDEN (Department of Bacteriology, Sheffield University, Sheffield 10)

Lester Smith (1942) showed that the silica gel partition chromatogram could be used to separate the lower fatty acids, C_1 - C_6 inclusive, chloroform—*n* butanol mixtures were the solvents, and bromo cresol green was the indicator used. This procedure was subsequently put on a quantitative basis (Elsden, 1946) with but minor modifications of the procedure outlined by Lester Smith. For convenience, formic acid was excluded from the analysis and acetic acid was estimated by difference, acetic, propionic and butyric acids could always be determined accurately and, with exceptional gels, valeric could also be estimated.

Recently, two groups of workers have extended the method to include valeric, hexoic, heptylic and octanoic acids, Scarisbrick, Baldwin & Moyle (1948) used heavily buffered, silica gel columns and chloroform—*n* butanol mixtures as the developing solvents. Peterson & Johnson (1948) used Celite 545 as the carrier with 30 *N* H_2SO_4 and benzene as the polar and non polar phases respectively. In both modifications the columns are, of necessity, 'run blind', and both therefore necessitate a large number of titrations for each estimation.

Isherwood (1946) applied the silica gel column, the separation and quantitative estimation of oxalic, malic, fumaric, succinic, tartaric and citric acids. The polar phase was made acid (0.5 *N* HCl) to suppress ionization of the acids and thus to suppress the tails of the bands, chloroform—*n* butanol mixture the developing solvents. The columns were 'run blind', but an ingenious technique, involving thymol blue as external indicator, was employed to facilitate making the cuts. Special precautions to be taken in the preparation of the gel. Lugg & Overell (1947) have separated a somewhat similar mixture of acids on paper, in this case formic acid was incorporated in the polar phase to suppress ionization, as yet the paper method is not quantitative.

The separation of purine and pyrimidine bases on paper and their subsequent estimation has been developed by Vischer & Chargaff (1947, 1948) and by Hotchkiss (1948), thus paving the way to the quantitative study of nucleic acids and their derivatives. Crammer (1948) has described a method for the separation of flavine nucleotides.

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Anthocyanins and Flavones By E. C. BATE SMITH (Low Temperature Research Station, University of Cambridge)

In an earlier note (Bate Smith, 1948) some details were given of the chromatography on filter paper of cyanin, peonin, malvin and hirsutin, of the corresponding monoglucosides and anthocyanidins, of the flavone apin and its hydrolysis products, and of a constituent of dahlia petals presumed to be the chalcone butein.

It was pointed out that the relative distance travelled by the anthocyanins in butanol acetic acid (the R_f value) is markedly affected by hydrochloric acid, but that there are, nevertheless, compensating advantages in the presence of this acid in the system. This effect of hydrochloric acid has, therefore, been explored in detail. A second solvent (phenol 1% acetic acid) has been found to give satisfactory results with many of the substances.

Anthocyanins In addition to those already named, samples of synthetic pelargonin and delphin, and of natural pelargonidin, cyanidin, peonidin and delphinidin 3 monosides have been obtained.*

The anthocyanidins have been prepared (in solution) by acid hydrolysis of the anthocyanins. The aglucones, especially cyanidin, are unstable when run on filter paper unless considerable hydrochloric acid is present. In HCl free butanol acetic acid they may disappear completely after several hours' running. Up to the present greatest stability has been obtained with butyl alcohol equilibrated with 2*N* HCl.

Fission products of anthocyanins When viewed in ultraviolet light, or when treated with ammoniacal

* From Mrs R. Meares.

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silver nitrate as for the development of reducing sugars, chromatograms of acid hydrolyzed anthocyanins show numerous spots which from their R_f values and from their relation to the structure of the particular anthocyanins can be attributed to products of the fission of the pyrilium ring. The identification of these as derivatives of catechol, phloroglucinol, pyrogallol, etc., is proceeding.

Flavones A considerable number of pure flavones and flavone glycosides have been examined. Regularities are apparent in the relationship between structure and R_f value in butanol acetic acid. With increasing OH, the R_f falls. (Quercetagenin has an R_f value which brings it within the range of the anthocyanins.) With increasing glycosidation, except in the case of rhamnosides, the R_f also regularly falls, but the position of the sugar residue affects considerably the extent of the fall. The monosides of the more usual flavones still fall well outside the range of the anthocyanins, but the biosides and, it may be anticipated, the diglycosides fall within this range. (As was shown, this question is important in the deciphering of chromatograms of plant extracts.)

The isoflavones irigenin and tectorigenin and their glycosides have similar R_f values to the

corresponding flavones and flavone glycosides, but their position on the chromatogram can only be detected by spraying with dilute aqueous FeCl_3 .

Chalcones Hydroxychalcones, including butein, have R_f values similar to those of the flavones of corresponding OH content. Butein occurs in the flowers of Compositae, as a rule, as mono- and diglycosides, the former (in butanol acetic acid) with R_f approximately 6, the latter, approximately 4, within the anthocyanin range.

Other polyphenolic substances The simpler polyphenols and their methyl and carboxylic acid derivatives show a great regularity in R_f value with increasing substitution. These substances can readily be demonstrated by developing with ammoniacal silver nitrate (those with ortho or para dihydroxy substitution reducing in the cold) or by spraying with dilute aqueous ferric chloride. A number of constituents of plant extracts have already been identified by this means, and their identity confirmed by simultaneous chromatography of the pure substances.

Chromatograms of extracts of flowers of Compositae, *Iris* and *Pelargonium*, were shown and discussed.

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Partition Chromatography with Stationary Phases other than Water Alone BY A. A. LEVI (Imperial Chemical Industries, Limited, Manchester)

Three main types of partition chromatogram are described in which water alone is replaced by other stationary phases. The first and most generally useful employs buffer solutions. The advantages and disadvantages of this type of column are discussed, and its use in the separation and purification of the penicillins is described. The technique should be useful in several other fields. A second type of column uses a solution or suspensions of a substance which reacts differentially but irre-

versibly with the components of a mixture. This type of column cannot give complete separations, but is valuable as a preliminary concentration of the constituents of a complex mixture. By suitable choice of stationary phase comparatively large quantities of material can be conveniently treated. Finally, a few systems can be used in which both stationary and flowing phase are non-aqueous. This method, although of restricted application, has given very useful results in particular problems.

General Review of the Applicability of the Method and Chairman's Summing Up BY R. L. M. SYNGE (Rowett Research Institute, Bucksburn, Aberdeenshire)

In the course of summing up, the following topics were briefly discussed:

- (i) Problems of the relationship between solvents, solute and 'supporting structure' and of adsorption phenomena in partition chromatograms
- (ii) Limitations on the molecular size of substances undergoing separation on partition chromatograms
- (iii) Possibilities for partition chromatography with the stationary phase the less polar one

(iv) The proper fields of application of partition chromatography and counter-current liquid extraction methods such as those of Craig and co-workers

(v) Molecular characteristics influencing the choice between adsorption and partition methods, improvement of separations by introducing extraneous reagents

(vi) Implications for biochemistry of the specificity of intermolecular interactions revealed in chromatographic studies

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The 271st Meeting of the Society was held in the Middlesex Hospital Medical School, London, W 1, on Saturday, 4 December 1948, at 11 a.m., when the following papers were read

COMMUNICATIONS

The Enzymic Decomposition of Blood-Group Substances By M V STACK and W T J MORGAN (The Lister Institute of Preventive Medicine, London and Elstree)

The action of the enzymes present in the culture supernatant fluids of certain strains of *Cl. welchii* (type B) has been examined, using as substrates preparations of blood group substances of animal and human origin (see Morgan, 1947). The enzyme activities can be differentiated by heating the enzyme material at 56° for 1 hr. whereby the activity against the group substances A and B is lost. The heated solutions, however, are able to inactivate the H serological character (Morgan & Watkins, 1948) of the so called 'O' substance (Morgan, 1946).

The unit of enzymic activity is defined as the amount of activity which will bring about the serological inactivation of 94% of 1 ml. of a 0.10% solution of the group substance in 2 hr. at 37° and pH 7.0. For practical purposes this is equivalent to an amount of inactivation which lowers the inhibition titre by four tubes when the usual geometrical dilution scale is employed.

Attempts to separate the enzymic activities by fractional precipitation techniques with ammonium sulphate or organic solvents, or by the addition of adsorbents to the supernatant culture fluids have been largely without success. Similarly, the adsorption on columns of adsorbents was examined but no useful separation of the enzymes was obtained. These procedures gave rise to a considerable purification

of the enzymes and activities of the order of 150 units per mg. N have been reached. The enzymic preparations are unstable unless stored dry at 0° or in glycerol solution at -10°. The optimum pH for the different activities measured was found to be between 5.5 and 6.5 (A, pH 5.5, H, pH 6.5), and no significant inactivation of the mucoids occurs at pH values below 4.0 and above 9.0. The A and H enzymes are more active at 45° than at 37° when tested over a 2 hr. period. The high viscosity shown by preparations of the mixed A and H mucoids derived from commercial pig gastric mucin (Morgan & King, 1943) is rapidly destroyed by an enzyme (depolymerase) present in the crude *Cl. welchii* filtrates. The depolymerase is inactivated after heating at 56° for 1 hr. and shows an optimum activity at pH 6.8. Solutions of potassium hyaluronate and purified human A and H substances are not rendered less viscous by this enzyme.

The lack of distinction between the physical properties of the enzymes is of interest and it could be surmised that certain groupings on the enzymic complex which are specific for the A or B substance or for the depolymerising activity, become denatured at 56° leaving other groups unaffected and still able to bring about the inactivation of the H serological character of the substrate.

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A Comparative Study of the Succinic Dehydrogenase-Cytochrome System in Heart Muscle and Kidney By E C SLATER (Molteno Institute, University of Cambridge)

Keilin & Hartree (1940) reported that kidney (as well as liver and other organs) did not contain the normal cytochrome system which is found in the actively respiring heart muscle, skeletal muscle and aerobic micro organisms. In the kidney preparation used by Keilin & Hartree, the bands of cytochrome c

and cytochrome b, found in heart muscle, etc., were replaced by a single wider band (with centre at 555m μ), which was believed to be the same as cytochrome b₁ found in certain micro organisms. In the presence of added cytochrome c, however, this preparation actively catalyzed the oxidation of

succinate This difference between heart muscle and kidney has been reinvestigated, using a different kidney preparation

It was found that kidney possesses essentially the same cytochrome system as is found in heart muscle and there is no evidence for the presence of cytochrome b_1 . It seems likely that the wide band observed by Keilin & Hartree is due to the presence of denatured protein haemochromogens in their preparation. Similarly, there appears to be no essential

Table 1 *Relative enzyme activities and concentrations of haematin compounds in heart muscle and kidney preparations*

All activities based on fat-free dry weight

	Heart muscle	Kidney	Heart muscle Kidney
Succinic oxidase (Q_{O_2})	625	200	3.1
Succinic dehydrogenase (Q_{O_2})	240	67	3.6
Cytochrome oxidase (Q_{O_2})	3200	1380	2.3
Protohaematin ($\mu M/g$)	1.8	1.8	1.0
Cytochrome c ($\mu M/g$)	0.8	0.27	3.0
Cytochrome b (μM haematin/g)	0.64	0.27	2.4
Catalase (μM haematin/g)	0.002	0.034	0.05
Cytochrome $a+a_3$ (arbitrary units)	1	0.54	1.9

difference between the succinic oxidase systems in the two tissues, except that the heart muscle preparation is the more active (Table 1)

Table 1 shows that both preparations contain unknown protohaematin compounds in addition to cytochrome b and catalase. The spectrum of these unknown haematin compounds is not directly visible in these preparations (cf Keilin, 1926, Slater, 1948)

The enzyme preparations are colloidal solutions of particles which are probably derived from some subcellular structure in the tissue. The Q_{O_2} (at 37°) of the cytochrome c in the heart muscle preparation is 38,000, which is half the activity of the cytochrome c in yeast (Keilin & Hartree, 1940). Cytochrome c added in solution is much less effective catalytically than the cytochrome c on the particles of the heart muscle preparation.

It was found that cytochrome b is involved in the reduction of methylene blue by succinate. It is impossible at present to choose between two possibilities: (1) cytochrome b is directly reduced by succinate, i.e. it is identical with succinic dehydrogenase, as has been suggested by Bach, Dixon & Zerfas (1946) and Ball, Anfinsen & Cooper (1947), (2) an additional enzyme, which is the true succinic dehydrogenase, is required for this reduction.

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β -Glucuronidase and Glucuronide Synthesis

(Department of Biochemistry, University of Edinburgh)

By M. C. KARUNARATNAM and G. A. LEVY

In view of the relationship observed between the activity of β glucuronidase in a tissue and the state of proliferation (Levy, Kerr & Campbell, 1948) it was considered important to find an inhibitor for this enzyme. Of many compounds studied, including several 'growth inhibitors', by far the most effective was saccharic acid. Almost complete inhibition of the hydrolysis of phenol glucuronide ($0.015 M$) by mouse liver or kidney preparations was obtained with $10 \times 10^{-3} M$ saccharate, and 50% inhibition with $2 \times 10^{-4} M$. Closely related compounds were much

less effective than saccharic acid. No evidence has so far been obtained of any action, even in massive doses, of saccharic acid on growth processes in the mouse.

It has been suggested, without any direct evidence, that β glucuronidase is responsible for glucuronide formation in the body (Fishman, 1940). Levy & Storey (1948) have developed a method for measuring glucuronide synthesis by mouse liver slices. In concentrations up to $10^{-2} M$, saccharic acid had no appreciable effect on this process.

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Obituary Notice

ALEXANDER THOMAS CAMERON, 1882-1947

Alexander Thomas Cameron, Professor of Biochemistry in the University of Manitoba, and an original member of the Biochemical Society, died at his residence in Winnipeg on 25 September 1947.

Born in London in 1882, of Scottish parentage, he received his early education in Swindon, proceeding thence to the University of Edinburgh from which he graduated M.A. in 1904 and B.Sc. in 1906. In the latter year he was awarded an 1851 Exhibition Scholarship, and, his interests lying in the field of physical chemistry, for the next two years he studied radiochemistry at University College, London, under Sir William Ramsay, followed by a year at the Technical High School, Karlsruhe, under Fritz Haber. In 1909 he was appointed Lecturer in Physiology in the University of Manitoba, an event which proved to be the turning point in his career.

With this appointment his attention was diverted to the ill defined field of physiological chemistry in which he speedily found problems with which his training and experience in the methods of physical chemistry enabled him to cope successfully. There, too, under the dynamic influence of Swale Vincent, he was introduced to the study of what were then known as the 'ductless glands', and commencing with an investigation of the distribution of iodine in plant and animal tissues, he laid the foundations of his reputation as an endocrinologist.

Except for a summer semester spent in research under Albrecht Kossel at the University of Heidelberg, and three years during the first World War when, as Captain, R.A.M.C., he acted as chemist officer for water purification with the British Expeditionary Force in France, his subsequent career was intimately linked with the University of Manitoba. The growing importance of physiological chemistry inevitably led to a separation from the parent department of physiology, and this was recognized by the institution in 1921 of a separate Chair of Biochemistry in the Faculty of Medicine, and the appointment of Cameron as Professor.

An indefatigable and painstaking worker, Cameron was author or joint author of over 100 published papers. His first paper, published while still a student at Edinburgh, appeared in the *Proceedings of the Royal Society of Edinburgh* in 1905. It dealt with the crystallization of potassium hydrogen succinate, and was followed by a series dealing with

physicochemical concepts, including the effect of heat and cold on cold blooded animals. His publications dealing with the biochemistry of iodine (for which he was awarded the degree of D.Sc. by the University of Edinburgh in 1915) were the forerunners of a long series on the effect of thyroid and thyroxine feeding on the growth and organ hypertrophy of the white rat. Another series of papers dealt with the biochemistry of calcium, hypoparathyroidism, and tetany, while other publications included the results of studies on creatine and creatinuria, pernicious anaemia, the production of tar carcinoma in mice, etc. The last of the long list was a monograph, the final proofs of which were submitted for his approval just prior to his death. It was entitled *The Taste Sense and the Relative Sweetness of Sugars and other Sweet Substances*, and embodied the results of some three years of experimental work in that little known field.

He was, however, best known for his text books. Never a very fluent lecturer, he recognized his limitations in that respect, and carefully prepared his lectures in biochemistry by writing them out in full. Since at that time there was no concise and authoritative text on Biochemistry, on the urging of his students and friends he submitted the manuscripts to a well known firm of publishers. Thus was born Cameron's *Textbook of Biochemistry*, the success of which is borne out by the fact that since its initial appearance in 1928 it has gone through six editions, as well as a Chinese and two Spanish editions. It was followed two years later by *Practical Biochemistry* (Cameron & White), now in its fifth edition. In 1933 two more books appeared, the result of his outstanding ability to present in clear and concise form a comprehensive and critical appreciation of a subject. These were *Biochemistry of Medicine* (Cameron & Gilmour) and *Recent Advances in Endocrinology*, of which the latter is now in its sixth edition and has been translated into Italian and Roumanian.

Fully occupied though he was with his teaching, writing, and research work, Cameron still had time for the public duties which fell to him as Chairman of the Fisheries Research Board of Canada, a position he held with conspicuous success for thirteen years. In this capacity he was chosen as one of the Canadian delegates to the Empire Scientific Congress held in London and Oxford in the summer of

1946 While attending these meetings he received official notification that his services had been recognized by the award of the C M G

Cameron was a Fellow of the Royal Society of Canada, a Fellow of the Royal Institute of Chemistry, a Past President of the Canadian Institute of Chemistry (now the Chemical Institute of Canada), and a member of various chemical, biochemical, and medical societies. One of his life long interests was the Scientific Club of Winnipeg, of which he was

of the early members, for twelve years its Secretary, and to whose Scientific Proceedings he made 46 contributions

Although of a very reserved nature, Cameron made friends in all parts of the Dominion, and they, together with many others to whom he was only known by name, mourn the passing of a scientist and scholar

F D WHITE

J B COLLIP

A Note on the Estimation of Vitamin B₁ in Urine

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(Received 6 October 1947)

The accurate measurement of the vitamin B₁ content of urine by the thiochrome method is made difficult by the interference of non specific fluorescent substances. Adsorption on the zeolite Decalso (The Permutit Co Ltd) as described by Hennessy & Cerecedo (1939) removes some of these, but not nicotinamide methochloride which occurs in the urine of man and some other animals including the rat. Treatment with alkali, ferricyanide and isobutanol in the thiochrome procedure converts nicotinamide methochloride to a violet fluorescing compound (F_3) (Coulson, 1944), which is indistinguishable fluorimetrically from thiochrome. When ferricyanide is omitted, another substance (F_2) with a bluish white fluorescence is formed from nicotinamide methochloride (Najjar & Holt, 1941). This makes the so-called 'NaOH blank' unsatisfactory. Several ways of overcoming this difficulty have been proposed (Mason & Williams, 1942, Najjar & Ketron, 1944, Coulson, 1944, Mickelsen, Condiff & Keys, 1945), but for reasons which will be given subsequently we believe that none of them is entirely satisfactory.

During work on the effect of sulphonamides on the excretion of vitamin B₁ by rats, we developed a procedure for avoiding interference by nicotinamide methochloride which, in our opinion, is an improvement on those at present in use. It depends on the fact that the blue fluorescence of thiochrome disappears on addition of acid to the isobutanol extract, whereas the fluorescence of F_3 is unaffected. By measuring the fluorescence before and after the addition of acid, a measure of the fluorescence due to thiochrome is obtained.

METHOD

A sample of urine (2–20 ml), containing if possible 2–3 μ g vitamin B₁, is adjusted to pH 4.5 with glacial acetic acid, and diluted to 50 ml with glass distilled water. One 25 ml portion is poured on to a Decalso column 5 cm long and 0.6 cm in diameter, 1.3 μ g vitamin B₁ is added to the other 25 ml portion which is poured on to another column. The shape and size of the adsorption tube and the activation of the zeolite are as described by Hennessy (1941). The columns are washed three times with 10 ml water, and the vitamin B₁ is then eluted with successive portions of 3, 3 and 5 ml 25% (w/v) KCl in 0.1N HCl. The volume of the eluate is adjusted to 11.0 ml.

For oxidation to thiochrome, 40% NaOH (1 ml) freshly prepared 1% K₃Fe(CN)₆ (0.1 ml) and redistilled isobutanol (15 ml) are added to a 5 ml portion of the eluate in a glass stoppered 2 oz. bottle. The contents of the bottle are well mixed after each addition and allowed to stand for 1 hr. If measurement of the fluorescence of F_2 is desired, a second bottle may be prepared in the same way except that ferricyanide is omitted, but this is not part of our routine procedure. Otherwise, the second 5 ml portion of the eluate may be used for a duplicate determination of vitamin B₁.

Measurement of the fluorescence in a Cohen type instrument (Henry, Houston, Kon & Osborne, 1939) is made with a 10 ml portion of the isobutanol layer. While this is still in the test tube of the fluorimeter, seven drops of a mixture of three parts methanol and four parts N HCl are added, the contents mixed, and the fluorescence measured again. The difference between the two readings is a measure of the fluorescence of thiochrome. This procedure makes the 'NaOH blank' unnecessary for the measurement of vitamin B₁, but useful as an indication of the amount of nicotinamide methochloride present.

It should be mentioned that this technique cannot be used with extracts containing methanol, which markedly increases the solubility of alkali in the isobutanol phase, as addition of sufficient HCl to neutralize this alkali leads to turbidity

RESULTS

The results of an experiment, in which pure substances were used (Table 1), show that the fluorescence of thiochrome, as observed visually and as measured fluorimetrically, disappears after treatment with acid whereas that of F_2 does not. The galvanometer deflexion due to thiochrome, obtained by difference, is almost the same for vitamin B₁ alone as in the presence of nicotinamide methochloride. Use of the 'NaOH blank' (F_2) would give a negative result for vitamin B₁ in the presence of nicotinamide methochloride. In our fluorimeter the fluorescence of F_2 appears greenish instead of bluish white.

Typical results obtained with normal human urine, and with urine of normal and vitamin B₁ deficient rats, are given in Table 2. Two assumptions are unavoidable in the application of our method, as of many other methods for the estimation of vitamin B₁ in urine. One is that vitamin B₁ is adsorbed and eluted to the same extent from the zeolite columns, through which the unknown with and without added vitamin B₁ had been passed, the other is that thiochrome is extracted to the same degree from eluates with and without added vitamin B₁. On the other hand, the measurement of the blank with the same isobutanol extract used for measurement of the unknown obviates two other possible sources of error: (a) that due to differences in the extent to which nicotinamide methochloride is adsorbed on, and eluted from, different zeolite columns, and (b) that due to differences in the extent to which F_2 and other fluorescent substances (not thiochrome) are ex-

Table 1 The effect of HCl treatment on the fluorescence of thiochrome and of F_2

Treatment	Substance and quantity taken for zeolite adsorption					
	Vitamin B ₁ (2.57 µg)		Nicotinamide methochloride (250 µg)		Vitamin B ₁ (2.57 µg) + nicotinamide methochloride (250 µg)	
	Galvano meter deflexion*	Colour of fluorescence	Galvano meter deflexion*	Colour of fluorescence	Galvano meter deflexion*	Colour of fluorescence
(1) NaOH, then isobutanol extraction	24	Not visible	228	Greenish	218	Greenish
(2) NaOH ferrieyanide, then isobutanol extraction	138	Blue	102	Violet	207	Bluish violet
(3) isobutanol extract (2) treated with HCl	35	Not visible	103	Violet	102	Violet
Deflexion due to thiochrome ((2) - (3))	103	—	—	—	105	—

* Linear scale

Table 2 The determination of vitamin B₁ in rat and human urine by the thiochrome method involving the use of HCl

(Values are given as galvanometer deflexions, linear scale)

Treatment	Rat urine				Human urine	
	Normal diet		Vitamin B ₁ deficient diet		Without added vitamin B ₁ †	With 1.3 µg added vitamin B ₁ †
	Without added vitamin B ₁ *	With 1.3 µg added vitamin B ₁ *	Without added vitamin B ₁ †	With 1.3 µg added vitamin B ₁ †		
(1) NaOH, then isobutanol extraction	142	140	320	360	364	374
(2) NaOH and ferrieyanide, then isobutanol extraction	236	300	79	158	161	233
(3) isobutanol extract (2) treated with HCl	58	56	80	89	76	77
Deflexion due to thiochrome ((2) - (3))	178	244	—	69	85	156
Calculated vitamin B ₁ content of urine	2.7 µg/ml		0.0 µg/ml		0.16 µg/ml	

* 1 ml urine taken

† 4 ml urine taken.

‡ 10 ml urine taken.

Table 3 *Urinary excretion of vitamin B₁ by rats with different intakes of vitamin B₁*

Treatment	No of observations	Urinary excretion (μg /rat/day)	
		Mean	Range
Diet deficient in vitamin B ₁	7	0	0-0
Diet deficient in vitamin B ₁ + 20 μg vitamin B ₁ daily	11	0.36	0.1-0.6
Diet deficient in vitamin B ₁ + 40 μg vitamin B ₁ daily	3	1.89	1.3-3.0

Table 4 *A comparison of the determination of vitamin B₁ in pure solutions in the presence and absence of nicotinamide methochloride by the method of Mickelsen et al (1945) and by our method*

(Values are given as galvanometer deflexions, linear scale)

Treatment	Substance and quantity taken for zeolite adsorption		
	Vitamin B ₁ (1.3 μg)	Nicotinamide methochloride (250 μg)	Nicotinamide methochloride (250 μg) + vitamin B ₁ (1.3 μg)
(1) NaOH, then isobutanol extraction at pH 12	34	270	293
(1a) NaOH, then isobutanol extraction at pH 8-9.5	40	37	39
(2) NaOH and ferricyanide, then isobutanol extraction at pH 12	98	103	180
(2a) NaOH and ferricyanide, then isobutanol extraction at pH 8-9.5	97	108	175
(3) isobutanol extract (2) treated with HCl	37	103	116
Deflexion attributed to vitamin B ₁			
Method of Mickelsen et al ((2a) - (1a))	57	71	136
Our method ((2) - (3))	61	0	64

Table 5 *A comparison of measurement of vitamin B₁ in rat and human urine, by the method of Mickelsen et al (1945) and by our method*

(Values given as galvanometer deflexions, linear scale)

Treatment	Urine* of rats on vitamin B ₁ deficient diet		Normal human urine†	
	Without added vitamin B ₁	With added vitamin B ₁ (1.3 μg)	Without added vitamin B ₁	With added vitamin B ₁ (2.6 μg)
(1) NaOH, then isobutanol extraction at pH 12	400	400	142	150
(1a) NaOH, then isobutanol extraction at pH 8-9.5	39	40	27	28
(2) NaOH and ferricyanide, then isobutanol extraction at pH 12	148	225	68	158
(2a) NaOH and ferricyanide, then isobutanol extraction at pH 8-9.5	148	226	63	147
(3) isobutanol extract (2) treated with HCl	148	150	47	50
Method of Mickelsen et al ((2a) - (1a))				
Deflexion attributed to vitamin B ₁	109	185	36	119
Calculated vitamin B ₁ content of urine	0.45 $\mu\text{g}/\text{ml}$		0.11 $\mu\text{g}/\text{ml}$	
Our method ((2) - (3))				
Deflexion attributed to vitamin B ₁	0	75	21	108
Calculated vitamin B ₁ content of urine	0.0 $\mu\text{g}/\text{ml}$		0.06 $\mu\text{g}/\text{ml}$	

* 4 ml. urine taken

† 10 ml urine taken

tracted by isobutanol in separate samples of the extract. We have frequently observed that the blank obtained for urine with added vitamin B₁ is slightly higher than that for urine alone, which may be due to a greater elution of nicotinamide methochloride from the zeolite column in the presence of additional vitamin B₁.

Results similar to those in Table 2 have been obtained with human urine on many occasions, but our experience has been primarily with rat urine. Typical results (Table 3) show that the excretion of vitamin B₁ by rats, as determined by our method, is closely related to the vitamin B₁ content of the diet.

DISCUSSION

We believe that our procedure is an improvement on those of other workers for the following reasons. We are in agreement with Najjar & Ketron (1944) that nicotinamide methochloride is partly destroyed by sodium sulphite and that the procedure suggested by Mason & Williams (1942) gives too high results. Najjar & Ketron (1944) and Coulson (1944) allow for the interference of nicotinamide methochloride by assuming that the galvanometer deflexion due to F_2 in the NaOH blank bears a fixed relation to that due to F_3 in the unknown. No such assumption is necessary in our method as we are able to measure F_3 directly.

In the method of Mickelsen *et al* (1945) the isobutanol extraction is carried out at a pH 8.0-9.5, at which F_2 in the NaOH blank does not fluoresce, but the fluorescence of thiochrome in the unknown is unpaired. Table 4 confirms this but shows that this treatment does not affect F_3 , so that in the presence of nicotinamide methochloride both thio-

chromo and F_3 contribute to the fluorescence. Table 5 shows that for this reason the method of Mickelsen *et al* (1945) gives too high results when applied to rat and human urine, e.g. the urine of vitamin B₁ deficient rats was found by this method to contain 0.45 μ g vitamin B₁/100 ml, whereas none was found by our method. For the sample of human urine the discrepancy was less since less nicotinamide was present.

SUMMARY

A modification of the thiochrome method for the fluorimetric determination of vitamin B₁ in urine in the presence of nicotinamide methochloride is described. Since treatment of the isobutanol extract with HCl removes the thiochrome fluorescence, the difference between readings taken before and after this treatment is a measure of the vitamin B₁ content of the urine.

We wish to thank Dr A. C. Bottomley for preparing the nicotinamide methochloride.

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Aggregate Formation in Soil

1 INFLUENCE OF SOME BACTERIAL POLYSACCHARIDES ON THE BINDING OF SOIL PARTICLES

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For maximum crop production it is essential that the soil should have a good crumb structure and that the crumbs should be stable and resistant to the dispersing effects of rain, frost, etc. Aggregate formation facilitates cultivation, drainage and aeration, increases the moisture holding capacity of soil and reduces erosion. It also maintains sufficient cohesion in the soil to give anchorage to plants, and yet sufficient incoherence to facilitate root penetration and emergence of seedlings. Large additions of organic matter and the growth of grassland vegetation are

credited with having an ameliorative effect on the physical state of the soil, but there is considerable diversity of opinion as to the exact processes involved in the formation of water stable aggregates.

Stable structure formation under natural conditions is possibly a gradual process influenced by many factors. It is reasonable to expect that physical, chemical and biological agencies are involved. The biological agencies include plants, animals and micro organisms, and of these the micro organisms are of great importance in pro-

ducing cementing or stabilizing substances, and filamentous forms like fungi, actinomycetes and some algae may have a mechanical binding effect

Geoghegan & Brian (1946) reported that bacterial polysaccharides of the levan and dextran types have a marked aggregating effect on soil particles. Levans in which fructofuranose units are joined by 2,6 linkages are formed by a wide variety of aerobic organisms including plant pathogens. Dextrans, polyglucoses in which the units are joined by 1,6 glucosidic linkages, are formed by various species of *Leuconostoc* bacteria and possibly other organisms.

As a preliminary to finding how these bacterial products aggregate soil particles, exceptionally pure samples of levans and dextrans have been prepared and examined in some detail.

EXPERIMENTAL

Bacterial strains used An organism, isolated from garden soil and identified as a strain of *Bacillus subtilis*, was used for levan synthesis. Cultures of *Leuconostoc dextranum* (NCTC Cat no 3354) and *Leuconostoc mesenteroides* (NCTC Cat no 3351) were obtained from the National Collection of Type Cultures and used for dextran production.

Media used Media having the following composition were employed

A	B
KH ₂ PO ₄ 15 g	K ₂ HPO ₄ 10 g
NaNO ₃ 20 g	Na(NH ₄)HPO ₄ 4H ₂ O 49 g
MgSO ₄ 7H ₂ O 0.5 g	MgSO ₄ 7H ₂ O 0.5 g
KCl 0.3 g	KCl 0.5 g
Distilled water 1000 ml	FeSO ₄ 7H ₂ O 0.01 g
	Bactopeptone 20 g
	Distilled water 1000 ml

Sucrose was added to (A) in amounts sufficient to give 2, 4, 6, 8 and 10% (w/v) concentrations of sucrose in the final medium and to (B) to give 2 and 10% (w/v). The media were adjusted to pH 7.0, dispensed in litre quantities in 1500 ml. Erlenmeyer flasks and sterilized by autoclaving under 15 lb/sq in. pressure for 15 min. To avoid precipitation it was necessary to sterilize the MgSO₄ 7H₂O separately, and after cooling to add it to the rest of the cooled medium employing aseptic technique.

Production of levans The media were inoculated with the washings of 48 hr old agar slant cultures of the organism, and after 8–10 days' incubation at 27° large amounts of polysaccharides were formed. The gross impurities were removed by filtering the solutions through no. 1 Whatman filter paper and the liquids were concentrated *in vacuo*. Following centrifugation and dialysis, sufficient ethanol was added to give 70% (v/v) in the final solutions, and the precipitated polysaccharides were obtained as white products. These crude materials, resuspended in water, formed viscous opalescent solutions, which contained large amounts of bacterial cells and 'cell debris'. In case nucleoproteins were present the solutions were adjusted to pH 4.0 and centrifuged. The precipitate was discarded and the supernatant fluid dialyzed until it had a pH of 7.0. Finally, proteins and bacterial cells were removed by the method

of Sevag (1934) and the purified levans were precipitated with ethanol, dried, and ground to fine powders.

Production of dextrans The media were inoculated with 5 ml of 4 day old sucrose broth cultures of the organisms and, except in the case of the product from *Leuconostoc dextranum*, the method of isolation and purification of the dextrans was the same as for levans. The dextran produced by *L. dextranum* was insoluble in water after ethanolic precipitation, hence in the isolation of this polysaccharide the first precipitation with ethanol was omitted.

Hydrolysis of levans The purified levans were dissolved in excess 0.1N H₂SO₄ and heated under reflux for 30 min. The hydrolysates were cooled to 60° and neutralized with solid BaCO₃, rapidly cooled and the BaSO₄ removed by centrifugation. The clear solutions were then made up to the required volumes.

Hydrolysis of dextrans The method of hydrolysis in this case was identical with that used for levan, except that 2N H₂SO₄ was used and heating was continued for 3 hr.

Estimations

(1) **Total reducing sugars** According to the Shaffer-Somogyi method (Heinze & Murneek, 1940).

(2) **Fructose** By the 'ferrocyanide method' of Becker & Englis (1941). Methyl red was used as indicator instead of sodium diphenylamnesulphonate.

(3) **Total N** By the micro Kjeldahl method (Pregl, 1930).

(4) **Aggregating effect** A soil, the structure of which in terms of water stable crumbs is extremely poor, was air dried and ground to pass through a 52 mesh BSS sieve. The polysaccharides, in colloidal suspension, were applied so that different samples of soil contained from 0.05 to 0.75% polysaccharide. Sufficient moisture was used to bring the soil to the 'sticky point'. In this state it was well stirred with a glass rod, placed on a 3 mm sieve and pressed through it.

The aggregates were dried at 50° and their stability was tested by the following technique. 25 g of aggregates were placed in 600 ml of water in a 1 l cylinder. The cylinder was inverted once and then allowed to stand for 30 min. After this the cylinder was inverted 30 times, the operation taking about 1 min. The contents were then poured on to a 52 mesh BSS sieve (8 in diameter) standing in a shallow bowl. Extra water was added to bring the level above that of the soil on the sieve. The sieve was then moved up and down 30 times, the water allowed to drain out each time, the sieve was gently tilted each time. The sieve was then removed, the contents washed into a basin and the whole evaporated to dryness (105°). The weight of water stable aggregates is expressed as a percentage of the air dried soil. The control samples (moistened with water, granulated and dried) were completely unstable when they were wet-sieved. Apparently, wetting and drying or pressing of moist soil through a sieve, *per se*, is unable to produce water stable aggregates.

The advantages of the above method are that there is very good agreement between replicates, results are reproducible and small differences in the aggregating abilities of the polysaccharides are easily measured.

Viscosity measurements To avoid terminological confusion, so common in this field, we adopted throughout the symbols and nomenclature proposed by Cragg (1946).

The measurement of viscosities of levan and dextran solutions involved the determination of the rate of flow of the solutions in an Ostwald viscometer. All solutions were thoroughly centrifuged before being run into the viscometer. The flow time and density (pyknometer method) of levan and dextran solutions, varying in concentration from 1.5 to 0.125% (w/v), and weaker dilutions where necessary, were determined at $20 \pm 0.1^\circ$, and from the data the relative viscosity (η_r) was calculated.

If d_1 = density of solvent,

d_2 = density of polysaccharide solution

t_1 = flow time of solvent,

t_2 = flow time of polysaccharide solution

then $\eta_r = \frac{t_2 d_1}{t_1 d_2} = \frac{t_2 d_1}{t_1}$ when the solvent is water, and the specific viscosity (η_{sp}) = $\eta_r - 1$

Hence the reduced viscosity (η_{sp}/c) may be calculated, and if this is plotted against the corresponding concentration c (in primary moles) the intrinsic viscosity

$$\lim_{c \rightarrow 0} \left(\frac{\eta_{sp}}{c} \right)$$

is obtained

RESULTS

Bacillus subtilis was cultured in a number of different media in which the sucrose concentration and source of nitrogen were varied, and in every case there was marked opalescence and an increase in viscosity associated with the formation of a polysaccharide which was a levan (97-98% fructose after hydrolysis). The levan from each medium was thoroughly purified, and several samples of the products from 2 and 10% (w/v) sucrose media were prepared.

Leuconostoc dextranum when cultured in media A and B containing 10% (w/v) sucrose produced large quantities of dextran (96-98% glucose) while *L. mesenteroides*, being apparently more fastidious in its requirements, produced dextran in medium B only. After ethanolic precipitation the dextran from *L. dextranum* was insoluble in water, while the product from *L. mesenteroides* gave a colloidal suspension similar to that of levans. However, when the material was worked into a paste and then diluted by adding more water a suspension was obtained which was fairly stable on standing, but precipitation occurred when it was centrifuged at a low speed.

Aggregation of soil particles

The ability of each product to form water stable soil crumbs was estimated. Results are presented in Figs 1, 2 and 3. The amounts of the different levans and dextrans applied to soil are expressed as a percentage of the air dried soil and the plotted values of percentage aggregation are the means of duplicate results, except in the case of levans 107 and F7 where only single estimations were made. For substances giving an average aggregation of 10-80%, s.e. (single determination) = ± 2.46 , and s.e. (mean

of 2 determinations) = ± 1.71 . For aggregations less than 10% or greater than 80%, s.e. falls towards zero.

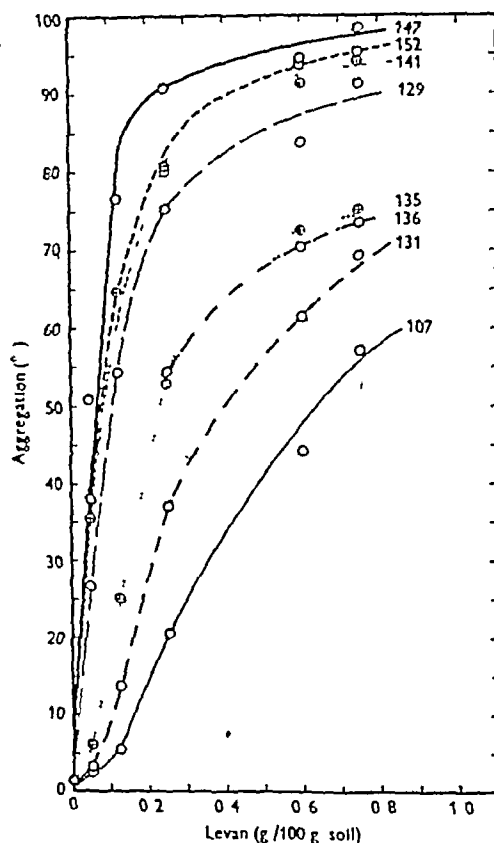


Fig 1 Effect of increasing quantities of various levans on aggregation of soil particles (Levans produced by *B. subtilis* when cultured in medium A containing from 2 to 10% sucrose)

Levan preparation	Culture medium
147, 152, 129	A + 2% (w/v) sucrose
141	A + 4% (w/v) sucrose
136	A + 6% (w/v) sucrose
135	A + 8% (w/v) sucrose
131, 107	A + 10% (w/v) sucrose

Up to 0.25%, the aggregating effect bears an approximately linear relationship to the amount of levan or dextran added. Consequently, 0.25% appears to be the most suitable concentration at which to appraise the aggregating effect of the different products.

All the levans and dextrans had a marked ameliorative effect on the physical condition of soil, but some levan products had a more pronounced effect than others. Apparently the aggregating effect of the levan decreases with increase in sucrose content of the medium. Also it appears that, when two or

more samples of levan are prepared from the same medium but from different lots of culture solution, their aggregating effects are not exactly alike

absorb a certain amount of levan, and, having absorbed this amount, the degree of aggregation produced is dependent on the activity of the substance

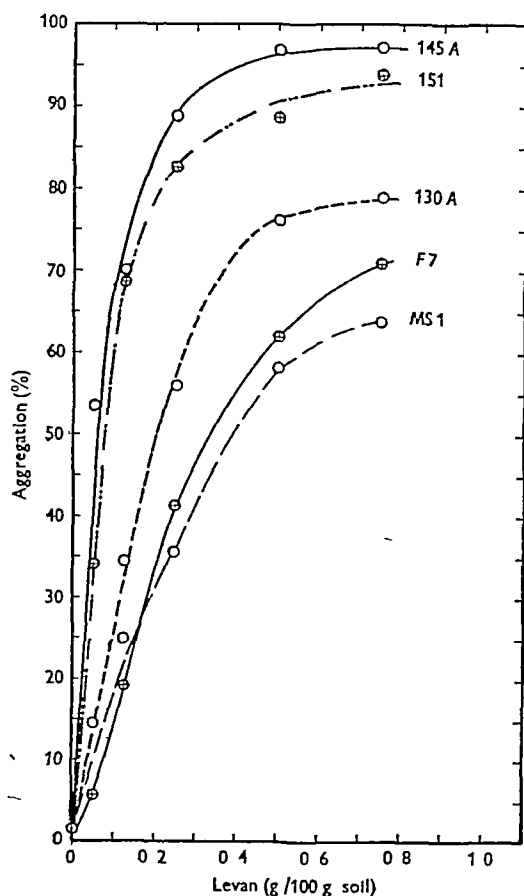


Fig 2 Effect of increasing quantities of various levans on aggregation of soil particles (Levans produced by *B subtilis* when cultured in medium B containing 2 and 10% sucrose)

Levan preparation	Culture medium
130 A, 145 A	B + 2% (w/v) sucrose
MS 1, F7, 151	B + 10% (w/v) sucrose

Effect of applying large doses of levan to soil In the case of those levans which possess a relatively low aggregating effect, it was thought that the degree of aggregation might be increased by applying larger quantities of levan to the soil. This was investigated (Table 1) by treating samples of soil with product 131, until the soil contained 2 and 5% levan, and comparing the degree of aggregation produced with that obtained when the soil contained from 0.05 to 0.75% levan. All determinations were done in duplicate and mean results are shown.

Apparently, further application of levan beyond a certain point has very little effect on aggregation of the soil particles, which suggests that soil can only

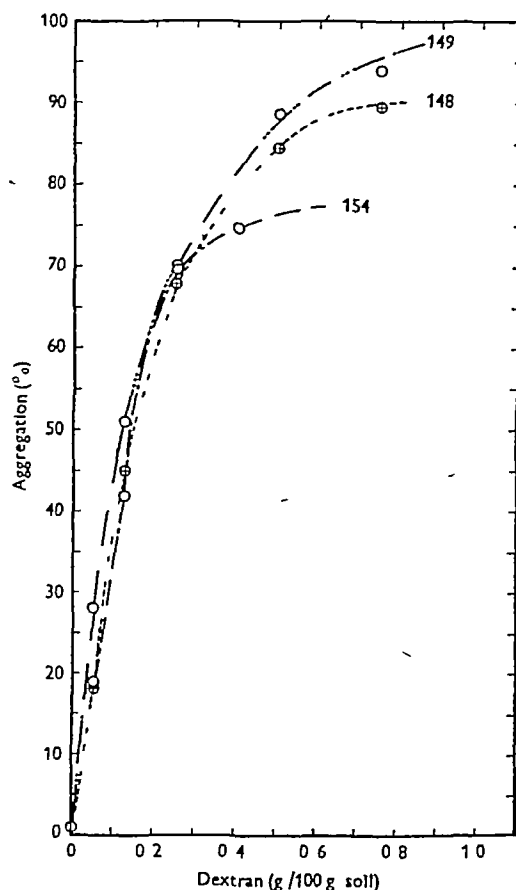


Fig 3 Effect of increasing quantities of various dextrans on aggregation of soil particles

Dextran preparation	Synthesizing organism	Culture medium
148	<i>Leuconostoc dextranicum</i>	B + 10% (w/v) sucrose
149	<i>Leuconostoc dextranicum</i>	A + 10% (w/v) sucrose
154	<i>Leuconostoc mesenteroides</i>	B + 10% (w/v) sucrose

Table 1 Effect of applying levan to soil in large and small amounts

Levan (no)	Amount added to soil (%)	Aggregation (%)
131	5.0	76.1
	2.0	65.6
	0.75	69.4
	0.50	61.4
	0.25	37.0
	0.125	13.6
	0.05	3.3
	Control	0.8

Table 2 *Effect of medium and of percentage sucrose in the medium on the intrinsic viscosity and molecular weight of levans produced by B subtilis*

($K_m = 5 \times 10^{-4}$)

Polysaccharide (no)	Medium in which polysaccharide was produced	Concentration c (in primary moles)	Flow time (sec)	Density d	$\frac{\eta_{sp}}{c}$	Mol wt
147	A + 2% (w/v) sucrose	0.093	286.6	1.007	36.2	26,800
		0.062	158.8	1.005	23.0	
		0.031	97.5	1.003	15.5	
		0.0155	79.8	1.001	13.4	
		0.0093	74.2	1.000	13.4	
			(Water=66.0)			
152	A + 2% (w/v) sucrose	0.093	169.6	1.007	17.0	18,800
		0.062	117.3	1.005	12.7	
		0.031	80.5	1.003	10.13	
		0.0155	75.8	1.001	9.68	
		0.0078	70.9	1.000	9.48	
			(Water=66.0)			
129	A + 2% (w/v) sucrose	0.093	112.4	1.007	14.30	16,000
		0.062	82.4	1.005	11.43	
		0.031	61.6	1.003	9.00	
		0.0155	54.3	1.001	7.70	
		0.0093	51.5	1.000	8.38	
			(Water=48.5)			
141	A + 4% (w/v) sucrose	0.093	131.0	1.007	10.73	14,000
		0.062	99.8	1.005	8.40	
		0.031	80.6	1.003	7.26	
		0.0155	73.4	1.001	7.28	
		0.0093	70.2	1.000	6.90	
			(Water=66.0)			
136	A + 6% (w/v) sucrose	0.093	71.1	1.007	5.12	8,000
		0.062	62.2	1.005	4.70	
		0.031	54.7	1.003	4.23	
		0.0204	52.4	1.002	4.00	
			(Water=48.5)			
135	A + 8% (w/v) sucrose	0.093	67.5	1.007	4.31	7,200
		0.062	60.2	1.005	4.00	
		0.031	53.9	1.003	3.71	
		0.0155	51.3	1.001	3.80	
			(Water=48.5)			
107	A + 10% (w/v) sucrose	0.083	56.4	1.006	4.42	6,400
		0.062	50.1	1.005	3.44	
		0.031	45.6	1.003	3.20	
			(Water=41.5)			
131	A + 10% (w/v) sucrose	0.093	64.6	1.007	3.67	6,200
		0.062	58.6	1.005	3.45	
		0.031	53.0	1.003	3.10	
		0.0155	50.8	1.001	3.10	
			(Water=48.5)			
145A	B + 2% (w/v) sucrose	0.093	134.5	1.007	19.3	18,000
		0.062	90.1	1.005	14.0	
		0.031	64.3	1.003	10.65	
		0.0155	55.1	1.001	8.71	
		0.0093	52.8	1.000	9.46	
			(Water=48.5)			
130A	B + 2% (w/v) sucrose	0.093	101.5	1.007	11.80	15,600
		0.062	79.8	1.005	10.53	
		0.031	61.1	1.003	8.48	
		0.0155	54.2	1.001	7.68	
		0.0093	52.1	1.000	7.96	
			(Water=48.5)			
MS1	B + 10% (w/v) sucrose	0.093	76.8	1.007	6.39	9,400
		0.062	65.6	1.005	5.81	
		0.031	55.9	1.003	5.00	
		0.0155	52.0	1.001	4.77	
			(Water=48.5)			
F7	B + 10% (w/v) sucrose	0.083	65.5	1.006	7.15	9,600
		0.062	55.8	1.005	5.66	
		0.031	47.8	1.003	5.00	
			(Water=41.5)			
151	B + 10% (w/v) sucrose	0.093	121.8	1.007	9.23	12,200
		0.062	97.2	1.005	7.74	
		0.031	78.9	1.003	6.42	
		0.0155	72.2	1.001	6.13	
			(Water=66.0)			

Table 3 *Intrinsic viscosity and molecular weight of the dextran produced by Leuconostoc mesenteroides*

$$(K_m = 5 \times 10^{-4})$$

Dextran (no)	Medium in which dextran was produced	Concentration c (in primary moles)	Flow time (sec)	Density d	$\frac{\eta_{sp}}{c}$	Mol wt
154	B + 10% (w/v) sucrose	0.111	185.6	1.009	16.5	14,000
		0.055	100.7	1.004	9.65	
		0.028	81.1	1.003	8.29	
		0.014	72.5	1.001	7.14	
		0.007	69.2	1.000	7.00	

(Water = 66.0)

Viscosity measurements

Since the aggregating effects of levans from different media were not alike, it was thought that the ability to stabilize soil crumbs might be related to some chemical or physical property of the polysaccharides. This idea was supported by the observation that after éthanolic precipitation the products from '2% (w/v) sucrose' media were tough and when stretched showed pronounced recoil, while those from '10% (w/v) sucrose' medium were viscous and capable of being drawn into threads. The chain length of the polymers, which influences their physical properties, was therefore investigated by viscosity measurements.

The intrinsic viscosities of solutions of the various levans and the dextran produced by *L. mesenteroides* were determined, and the values correlated with the corresponding aggregating effects. Whilst the value $\lim_{c \rightarrow 0} \left(\frac{\eta_{sp}}{c} \right)$ yielded all the information required, the

molecular weights were found from the Staudinger relationship $\frac{\eta_{sp}}{c} = K_m M$, where K_m is a constant estimated to have the value 10^{-3} for cellulose in Schweitzer's reagent ($c = 1.4\%$ (w/v) (Staudinger, 1933)). More recently the constant $K_m = 5 \times 10^{-4}$ has been used by Staudinger (1938).

The constants for our levans and dextran were not determined and $K_m = 5 \times 10^{-4}$ was selected. The use of a constant which applies to cellulose in Schweitzer's reagent in calculating the molecular weights of levans and dextrans is admittedly an approximation but gives information on the order of the molecular weights. The results are shown in Tables 2 and 3.

The intrinsic viscosities and hence the molecular weights of the levans decreased with increasing sucrose content of the medium. In the case of '2% sucrose' media the form of nitrogen supplied to the organism did not materially influence the intrinsic viscosities of the products. The intrinsic viscosity of the dextran produced by *L. mesenteroides* is similar to that of some levans.

There appears to be a relationship between the value of $\lim_{c \rightarrow 0} \left(\frac{\eta_{sp}}{c} \right)$ and the percentage aggregation.

From the values for intrinsic viscosity and aggregating effect of 13 levans and 1 dextran a correlation coefficient was calculated and found to be 0.809. This value indicates a significant correlation (odds 1 in 1000).

A dot diagram, in which the value of $\lim_{c \rightarrow 0} \left(\frac{\eta_{sp}}{c} \right)$ is plotted against the degree of aggregation produced when soil contains 0.25% of the various levans and dextrans, is given in Fig. 4. No curve is fitted to the

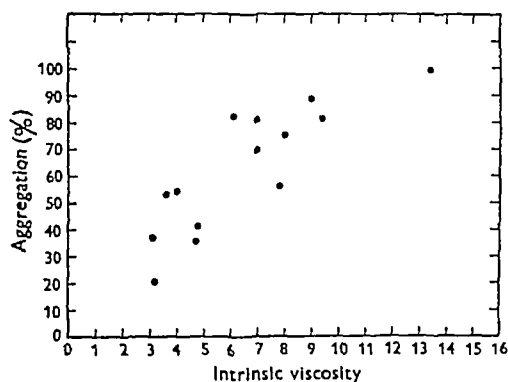


Fig. 4 Relationship between the intrinsic viscosities and the aggregating effects of 13 levans and 1 dextran

points on the diagram, this cannot be done satisfactorily until the shape of the curve as it approaches zero and 100% aggregation can be forecast for substances of lower and higher intrinsic viscosities than those so far considered.

Nitrogen content of levans and dextrans

Solutions of bacterial polysaccharides are very viscous and separation of the metabolic products from bacterial cells is very difficult. Levans and dextrans as usually prepared contain a small amount of nitrogen (0.2–0.5%) which may be present as an impurity or as a constituent of the polysaccharide.

molecule Stacey (1943) has suggested that dextrans consist of polyglucose chains linked together by units of the synthesizing enzyme which remains as an integral part of the complex mucopolysaccharide. In our earlier investigations we found that the levan obtained when *B. subtilis* was cultured in '10% sucrose' media contained 0.09% N, whereas that from '2% sucrose' media contained 0.2 to 0.3% N, and since the product from the '10% sucrose' media was not as effective an aggregator of soil as the product from '2% sucrose' medium it appeared that the aggregating effect was related to the nitrogen

increases with increase in sucrose content of medium A. This increase in yield of levan is accompanied by a lowering of the nitrogen content of the 'crude levan', which suggests that the ratio of levan to bacterial cells in the culture solution is increased with increase in sucrose content of the medium. Microscopical examination of levan solutions has shown that, even after most careful purification, a small amount of bacterial cells remains. It is reasonable to expect that the amount of bacterial cells that remains is influenced by the ratio of levan to bacterial cells in the crude material.

Table 4 Nitrogen content, molecular weight and aggregating effect of some levans and dextrans

Polysaccharide (no) -	Medium in which polysaccharide was formed	Nitrogen (%)	Mol wt	Aggregating effect when soil received 0.25% polysaccharide
Levan				
147	A + 2% (w/v) sucrose	0.12	26,800	90.9
152	A + 2% (w/v) sucrose	0.13	18,800	80.1
129	A + 2% (w/v) sucrose	0.05	18,000	75.1
130 A	B + 2% (w/v) sucrose	0.18	15,000	56.0
145 A	B + 2% (w/v) sucrose	0.09	18,000	88.9
141	A + 4% (w/v) sucrose	0.09	14,000	80.9
136	A + 6% (w/v) sucrose	0.06	8,000	54.1
135	A + 8% (w/v) sucrose	0.03	7,200	52.9
131	A + 10% (w/v) sucrose	0.02	6,200	37.0
107	A + 10% (w/v) sucrose	0.10	6,400	20.5
MS1	B + 10% (w/v) sucrose	0.10	9,400	35.8
F7	B + 10% (w/v) sucrose	0.09	9,600	41.4
151	B + 10% (w/v) sucrose	0.06	12,200	82.7
Dextran				
148	B + 10% (w/v) sucrose	0.22	—	67.9
149	A + 10% (w/v) sucrose	0.00	—	70.1
154	B + 10% (w/v) sucrose	0.11	14,000	69.7

content. However, by improving the technique used for separating the polysaccharides from the bacterial cells, the nitrogen content of the products was lowered appreciably without degrading the products and without lowering the aggregating effects. In Table 4 are shown the nitrogen content, molecular weight and aggregating effect of some of our most purified levans and dextrans.

It appears that the aggregating effects and the molecular weights of these products are not related to their nitrogen contents. This is most evident in the case of levans from the same medium.

In the case of levans 141, 136, 135 and 131 there appears to be a decline in molecular weight and aggregating effect as the nitrogen content of the products decreases, but these materials are from media containing different concentrations of sucrose, and it has been shown that the molecular weight and aggregating effect decrease with increasing sucrose concentration of the medium. The decline in nitrogen content, accompanying the decrease in molecular weight and aggregating effect, is possibly due to the fact that the yield of levan/l of culture solution in

In another experiment levans were isolated in crude form and samples of the crude material were purified to different degrees. The nitrogen content and molecular weight of each product were determined. Results are recorded in Table 5.

Table 5 Nitrogen content and molecular weight of 'crude' and 'purified' levans

Levan (no)	Nitrogen content (%)	Molecular weight
151 Crude	0.13	12,200
Purified	0.06	12,200
139 Crude	0.43	6,200
Purified	0.11	7,600

Relatively large amounts of nitrogen were removed without degrading the substances, and it appears that there is no correlation between molecular weight and nitrogen content of levans produced by *B. subtilis*.

Table 6 shows the results of an experiment designed to test how the aggregating effect of a levan was affected when its nitrogen content was lowered.

Table 6 *Relation between aggregating effect and nitrogen content of a levan*

Levan (no)	Amount added to soil (%)	Aggregation (%)	
		Sample 1, N=0.25%	Sample 2, N=0.09%
124	0.75	73.8	83.3
	0.50	69.8	78.6
	0.25	48.8	60.8

As the nitrogen content decreased the aggregating effect increased, which shows that purification removed only extraneous matter and did not affect the ability of the substance to form water stable soil crumbs.

It will be recalled that it has been suggested that polysaccharides owe certain of their immunological properties to the presence in them of a nitrogenous constituent, e.g. Fitzgerald (1933) found from serological studies that the antigenic activity of a polysaccharide produced by *Leuconostoc mesenteroides*

separate actions, and it is not necessary to degrade the levan to remove nitrogen.

Since the intrinsic viscosities, and hence the molecular weights of levans and dextrans, were not affected by removal of nitrogen, it is concluded that the nitrogen content of the polysaccharides examined is due to the presence of bacterial cells, as impurities, in the substances.

Stability of soil aggregates made with bacterial levan

It is well known that unstable soil aggregates disintegrate when they are placed in water. However, when aggregates containing 0.5% levan were submerged in water for 20 days it was observed that they were more or less unchanged. They had absorbed a large amount of moisture but showed no sign of crumbling.

The data in Table 7 show that the method of wetting aggregates containing levan does not materially influence their stability.

Table 7 *Effect of maintaining aggregates in a moist condition for long and short periods before wet sieving*

Levan (no)	Amount added to soil (%)	Aggregation (%)	
		Aggregates absorbing moisture by capillary action for 17 hr. before wet sieving	Aggregates submerged for 30 min. before wet sieving
151	0.5	84.7	88.5
	0.125	54.4	68.8

disappeared when its nitrogen content was reduced to less than 0.2%. However, Evans, Hawkins & Hibbert (1941) found that anti *L. mesenteroides* sera (produced in rabbits) gave precipitin reactions with relatively high dilutions of the homologous polysaccharide, dextran, having a maximum nitrogen content of 0.08%. This suggests that the small amount of nitrogen present in the dextran may not be responsible for the immunological reactions of the polysaccharide.

When our levans were treated with hot dilute NaOH, a flocculent precipitate appeared in the solution, and when it was removed a degraded levan was recovered from the solution which had a reduced nitrogen content and a very low aggregating effect (cf. Stacey, 1943). A similar precipitate was formed when solutions of the levan from *B. subtilis* were dialyzed. The precipitate settled to the bottom of the dialysis tube and was easily removed. Microscopic examination showed that it consisted, almost entirely, of bacterial cells. The levan recovered from the dialyzed solution had a much lower nitrogen content and a slightly higher aggregating effect. Apparently, the effect of NaOH on levan solutions is twofold: it facilitates removal of nitrogen by precipitating the bacterial cells and 'cell debris', and it degrades the levan. It is thought that these are

In another experiment aggregates containing 0.5% levan (product 151) were dropped into boiling water and held at a temperature of 95° for 10 min. with and without stirring. They were then cooled and the degree of aggregation measured by the wet-sieving technique. It is rather remarkable that, without stirring, a large proportion (over 65%) of the aggregates remained stable after heating, and that, with stirring, as much as 35% still remained stable.

Soil aggregates, containing 0.5% levan, were broken down when shaken in water for 1 hr. However, when the fine soil was removed from the shaking bottle, dried to the 'sticky point' and pushed through a 3 mm sieve, water stable aggregates were reformed. Approximately 40% of the polysaccharide applied to the soil was present in the water with which the soil was shaken. These results suggest that a certain amount of levan is fixed on soil particles.

Table 8 gives the results of an experiment designed to discover if levan, present on soil particles, was resistant to microbial decomposition. Aggregates containing 0.5 and 0.125% levan were moistened with a soil suspension and incubated at 27°. At intervals samples were removed and the percentage of water-stable aggregates estimated.

It is concluded that levan present on soil particles is slowly decomposed by micro organisms.

Table 8 *Effect of microbial activity on stability of 'levan soil aggregates'*

Levan (no)	Amount added to soil (%)	Aggregation (%)			
		Before incubation	After 5 days' incubation	After 11 days' incubation	After 31 days' incubation
151	0.5	88.5	82.8	61.0	44.1
	0.125	68.8	23.7	8.2	7.2

DISCUSSION

Polysaccharides are normal metabolic products of micro organisms. Numerous species of bacteria, fungi and actinomycetes are capable of producing a wide variety of complex polysaccharides as cell constituents or as extracellular products. Hence it is reasonable to expect that, under natural conditions, the diverse flora of the soil may synthesize many polysaccharides from the constituents of vegetable material, and that these substances may be of considerable importance in the formation of crumb structure in soil.

Polysaccharides are probably only one of the groups of microbial metabolic products having an ameliorative effect on soil structure, and only when further knowledge is available will it be possible to estimate the full extent to which biological activity influences the physical condition of soil.

SUMMARY

1 It has been shown that bacterial polysaccharides of the levan and dextran types exert a pronounced binding effect on soil particles.

2 *Bacillus subtilis* was cultured in a number of different media, in which the sucrose concentration

and source of nitrogen were varied, and it was observed that the yield of levan increased with increase in sucrose content of the medium from 2 to 10% (w/v). The aggregating effect of the levan decreased, however, with increasing sucrose content of the medium, and was paralleled by a decrease in intrinsic viscosity and molecular weight of the levans.

3 It appears that the nitrogen content of levans and dextrans is due to the presence of bacterial cells, as impurities, in the substances. There was no correlation between aggregating effect and nitrogen content, or between molecular weight and nitrogen content of either levans or dextrans.

4 Soil crumbs, containing small amounts of levan, are extraordinarily stable to the dispersing actions of water, heat and shaking, but are eventually broken down by the activities of micro organisms.

5 Preliminary investigations indicate that levans are fixed on soil particles.

6 It is not suggested that polysaccharides are the sole agents concerned in aggregating soil, but it does appear that they may be of considerable importance.

Grateful acknowledgements are due to Mr P. A. Collier for statistical analysis of the results and to Dr A. H. Lewis for helpful advice.

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Aggregate Formation in Soil

2 INFLUENCE OF VARIOUS CARBOHYDRATES AND PROTEINS ON AGGREGATION OF SOIL PARTICLES

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Geoghegan & Brian (1948) reported that bacterial polysaccharides of the dextran and levan types had a marked aggregating effect on soil particles. Further, it was observed that aggregating effect was related to the intrinsic viscosity and molecular weight of the substances. These results suggest that other viscous substances of high molecular weight may have an ameliorative effect on soil structure.

EXPERIMENTAL

The aggregating effect of various carbohydrates and proteins was determined by the method of Geoghegan & Brian (1948).

RESULTS

Results are presented in Table 1. The amounts of the different substances applied to soil are expressed as percentages of the air dried soil and the figures quoted for aggregation are the means of duplicate results.

Table 1 *Influence of various carbohydrates and proteins on aggregation of soil particles*

Substance	Percentage aggregation* when 0.25% of the substance was applied to soil
Crude polysaccharide from a strain of <i>Penicillium luteum</i>	50.1
'Cellofas W L D'	45.5
'Cellofas W F Z'	25.6
Quince gum	25.5
Pectin	3.4
Inulin	0.8
Egg albumin	32.9
Trypsin	4.5
Pepsin	1.2
Pancreatin	1.1
Control	1.2

* For substances giving an aggregation greater than 10%, S.E. for the mean of two determinations = ± 1.09 .

DISCUSSION

The crude polysaccharide from *Penicillium luteum* had a definite aggregating effect, although less than that of levans and dextrans (cf. Geoghegan & Brian, 1948). This indicates that under natural conditions fungal polysaccharides, like bacterial polysaccharides, may play an important role in soil structure formation.

The results obtained with egg albumin, trypsin, pepsin and pancreatin suggest that some proteins are capable of aggregating soil particles. Proteins from plants and micro organisms are present in soil organic matter, and it is possible that some of these substances may help in the formation of water-stable soil crumbs.

It is interesting to note that 'Cellofas W F Z', although more viscous than 'Cellofas W L D', is less effective as an aggregator, the former is the sodium salt of carboxymethyl cellulose and the latter an ether of cellulose.

Inulin, which is composed of anhydrofructofuranose units, the linkages being at positions 1 and 2 of the fructose chain, is unable to aggregate soil. This is not surprising, because solutions of this substance have low viscosities. Haworth (1936) claims that the inulin molecule consists of a laminated series of fructofuranose rings which are not joined end to end as in the case of cellulose or starch, and that this spatial arrangement is responsible for the low viscosity of solutions of the substance.

Pectin, the specific viscosity of which is greater than that of levans from *Bacillus subtilis*, failed to aggregate soil, and quince gum which is still more viscous only caused 25% aggregation. This is important as it shows that, while substances capable of aggregating soil have high viscosities, there is no reason to expect that all viscous substances will improve soil structure.

Work along these lines will provide information on the relative powers of different substances to aggregate soil particles, and will help to elucidate the relationship between chemical structure and the ability of a substance to form water stable soil crumbs.

SUMMARY

The aggregation of soil particles by viscous substances other than levans and dextrans has been assessed. Results show that while some biological products have a moderate aggregating effect it does not follow that all viscous substances will improve soil structure.

Our thanks are due to Dr G. G. Freeman for supplying samples of the polysaccharide from *Penicillium luteum* and to Dr A. H. Lewis for his constant interest and advice.

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Antipyretic Action and Catalase Activity

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Antipyretics are well known as stabilizers of commercial hydrogen peroxide solutions (Kausch, 1938, Slater, 1945), and it has been suggested by Bailev (1937) that 'antipyretic substances are in general aromatic, many of them phenolic, and quite possibly lower the temperature of the body by reducing the rate of oxidation', conceivably by interfering with H_2O_2 metabolism. In tissue metabolism, H_2O_2 may be produced by reactions involving, for example, amino acid oxidase, xanthine oxidase, uricase (Sumner & Somers, 1943), and the chemical identification of H_2O_2 as a metabolic product has recently been demonstrated by Tosic & Walton (1946). The H_2O_2 produced is decomposed by the widely distributed cellular enzyme catalase, but simple protective decomposition of H_2O_2 into O_2 and water is now considered by Keilin & Hartree (1945) to be less likely than catalysis of coupled oxidation of alcohols, and possibly of other biologically important compounds.

The possibility that antipyretic action may not be wholly central in origin is suggested by the hyperthermic action of dinitro- α -naphthol in a dog deprived of its central thermoregulatory mechanism (Heymans, 1928), and by the antipyretic action of acetanilide, quinine and pyrimidine in decerebrate rabbits exhibiting pyrexia induced by β -tetrahydro-naphthylamine (Skowronski, 1929). The antipyretic substances, sodium salicylate, quinine and pyrimidine, have a depressant action on isolated tissue respiration (Nitzescu & Cosima, 1923), and the reduction of methylene blue by liver tissue is inhibited by antipyretics such as aspirin, antipyrine, phenacetin, quinine and sodium salicylate, as well as by a number of typical antioxidants (Boutaric & Jacquinet, 1931).

Antipyretic activity is broadly associated with particular chemical structures, and several representative antipyretic substances were tested for inhibitory activity in the isolated catalase H_2O_2 system, in an attempt to provide evidence for the hypothetical mechanism suggested above for possible peripheral action of antipyretics. Of the four main types of antipyretic substances, acetanilide and phenacetin represent typical aniline derivatives, salicylic acid and acetylsalicylic acid represent typical phenol derivatives, antipyrine is a representative pyrazolone derivative, and quinine repre-

sents a number of quinoline derivatives. *p*-Hydroxybenzoic acid is a phenol derivative not ordinarily used as an antipyretic.

EXPERIMENTAL

A horse liver catalase extract, prepared by the method of Sumner, Dounce & Frampton (1941) had a 'Katalase f' value (determined by the method of von Euler & Josephson, 1927) of 2500. The catalase content was calculated by colorimetric estimation of the Fe content, by the method of Delory (1943), assuming 0.062% Fe in the dry enzyme (von Euler, Zeile & Hellström, 1930).

The test substances used were A.R. grade, further purified as follows: acetanilide, phenacetin, aspirin, salicylic acid and *p*-hydroxybenzoic acid (recrystallized from conductivity water), quinine (recrystallized from pure benzene), antipyrine (recrystallized from pure toluene). The pH of the Sørensen buffer was checked electrometrically. All solutions were prepared in glass distilled water, and dust was excluded as far as possible.

Reaction rates were determined at 37° using the Barcroft manometer. The right hand manometer flask contained 2 ml of 0.32M H_2O_2 (M.A.R.), and 2 ml of 0.0067M phosphate buffer, pH 6.8, the left-hand flask 2 ml of 0.32M H_2O_2 , 1 ml of 0.0067M buffer, 1 ml of a solution of the test substance, and a Keilin pipette holding 0.16 ml of undiluted catalase extract. For the calculation of percentage inhibitions, reaction rates were compared with that of the control in which the amount of O_2 evolved over a period of 5 min was taken as a measure of initial reaction velocity.

RESULTS

The percentage inhibitions obtained with the substances tested are given in Table 1. The observed inhibitions with the hydroxybenzoic acid derivatives can be due only in negligible degree to the slight lowering of pH, and probably result from the presence of phenolic OH groupings. The latter may interrupt the chain reaction, postulated by Sumner (1941), which involves the formation of the free radicals OOH and OH. A number of workers (e.g. Richter, 1934, Alyea & Pace, 1933, Yakushiji, 1937, Collier, 1940) have shown, in fact, that catalase is inhibited by phenolic compounds. Collier has shown that, in general, catalase is inhibited by phenols, oximes and amino derivatives which may give rise to HO-NH groupings on oxidation, and that these compounds produce methaemoglobin from haemoglobin, reduce Folin's phenol reagent,

Table 1 *Inhibition by test substances of the decomposition of 0.16M-H₂O₂ by catalase*

Conc of antipyretic (M × 10 ⁻³)	Percentage inhibitions						
	Acetanilide	Phenacetin	Salicylic acid	Acetyl salicylic acid	p Hydroxy-benzoic acid	Antipyrine	Quinine
0.4	—	—	—	—	—	—	0
1.1	—	0	44	17	—	0	—
1.8	0	—	—	—	47	—	—
2.3	—	—	76	42	—	—	—
2.6	—	—	—	—	—	0	—
3.5	0	—	—	—	68	—	—
4.5	—	—	89	70	—	—	—
5.3	—	—	—	—	—	0	—
7.0	0	—	—	—	—	—	—
10.5	—	—	—	—	—	0	—
14.0	28	—	—	—	—	—	—

give colour reactions with FeCl₃ and resemble anti-oxidants

Experiments with concentrations higher than those recorded for phenacetin and quinine were excluded by the low solubility of these compounds. With antipyrine there is obviously no correlation with antipyretic activity, and inhibition with acetanilide was only slight at relatively high concentrations. It is thus not possible to associate antipyretic activity in every case with the *in vitro* decomposition of H₂O₂ by catalase.

SUMMARY

1. Since many antipyretics can be used to stabilize H₂O₂ solutions, it has been suggested that they may also conceivably interfere with H₂O₂ metabolism. To test this theory, a number of representative antipyretics have been tested for inhibitory activity in the isolated enzyme system, catalase-H₂O₂.

2. Inhibition was observed with some, but not all, of the substances tested, i.e. there was no parallelism with antipyretic activity.

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Note on the Manometric Determination of Succinic and α -Ketoglutaric Acids

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Small quantities of succinic and α ketoglutaric acids, from 0.2 mg upwards, are now commonly determined manometrically with the help of succinic dehydrogenase (Szent Györgyi & Gözsy, 1935, Krebs, 1937, 1938). The object of this paper is to draw attention to two sources of errors which we have recently encountered.

Inhibition of succinic dehydrogenase by phosphotungstates

In some determinations of succinate it was noted that the ethereal extract contained a powerful inhibitor of succinic dehydrogenase, resulting in delayed or incomplete oxidation of the succinate. This was found to be phosphotungstate which arises from the tungstate added as a deproteinizing agent and from phosphate present in the tissue preparations. Phosphotungstic acids are known to be readily extractable with ether.

Whilst Na tungstate ($2 \times 10^{-2} M$) has no effect on succinic dehydrogenase, Na phospho 18 tungstate ('A acid' of Wu, 1920) is a strong inhibitor in relatively low concentrations. For example $0.5 \times 10^{-4} M$ caused an inhibition of 64% (pH 7.4, succinate concentration 0.01M, 40°). Na phospho 24 tungstate, also prepared according to Wu (1920), is likewise an inhibitor, but higher concentrations, about five times that of the 18 acid, are required to produce the same effect.

These inhibitions might be expected as it is known that succinic dehydrogenase possesses SH groups (Hopkins & Morgan, 1938), and that phosphotungstates readily oxidize SH compounds to the disulphides (Folin & Marenzi, 1929, Schöberl & Ludwig, 1937, Schöberl & Rambacher, 1938). The inhibition of succinic dehydrogenase depends on the concentration of succinate. This again is not unexpected since Hopkins, Morgan & Lutwak Mann (1938) have shown that succinate protects the SH groups of the enzyme against other reagents.

When solutions of tungstate which do not contain phosphate are acidified, practically all the tungstic acid is precipitated, but in the presence of phosphate, tungstic acid in excess of that reacting with protein and similar substances remains in the solution as complex phosphate. The formation of inhibitory

quantities of phosphotungstate can easily be prevented by avoiding an excess of tungstate. A minimum amount, ascertained on a sample of the material, is recommended. We find that 4 ml of a neutralized tissue suspension containing 0.4 g of fresh tissue and diluted to 15 ml with water or washings is satisfactorily deproteinized by the addition of 0.25 ml of 10% (w/v) Na tungstate and subsequent acidification with H_2SO_4 (blue to congo red). We retain tungstate as a deproteinizing agent because, unlike other deproteinizing agents, it yields filtrates which do not froth when ether is bubbled through the solution at a rapid rate.

Interference of glutamic acid in the determination of α ketoglutaric acid

$KMnO_4$ in acid solution is used for the oxidation of α ketoglutaric to succinic acid, and it has been pointed out previously (Krebs, 1938) that several substances which may occur in biological material, such as α hydroxyglutarate and arginine, also yield succinate when treated with the reagent. When these substances are present α ketoglutarate must be separated prior to the oxidations. This can be effected by the conversion into the 2,4 dinitrophenylhydrazone and ethereal extraction of the latter. In the absence of interfering material a simplified procedure may be adopted, i.e. direct oxidation with $KMnO_4$ of the deproteinized solution (Krebs, 1938, Krebs & Eggleston, 1940).

In recent experiments we found that under some conditions glutamic acid, which is frequently present in biological material, yields succinate on treatment with $KMnO_4$. It is noteworthy that in pure solution glutamic acid does not react significantly. Previous findings on this point (Krebs, 1938) were confirmed by recent tests, it was further found that no oxidation of glutamic acid occurred in mixtures of glutamic acid with α ketoglutaric acid, or glucose, or pyruvate, or lactate and several amino acids. But in deproteinized homogenates of liver and other tissues added glutamic acid was found to yield varying amounts of succinate, the maximum being 70% of the theoretically possible value. Deproteinized suspensions of minced liver had little or no effect on the oxidation of glutamic acid.

We have not been successful in identifying the substance in liver and other tissue homogenates responsible for the oxidizability of glutamic acid by acid KMnO_4 . The following substances added to a pure solution of glutamic acid had no effect on the oxidizability: CuSO_4 , FeSO_4 , MnSO_4 , haemin, the substances of rabbit bile, the amino acids contained in casein hydrolysate.

In view of the widespread occurrence of glutamic acid in biological material the direct treatment of deproteinized solutions with permanganate must be deprecated. We separate α -ketoglutaric from glutamic acid by extraction of the strongly acidified solution with ethyl ether, using a continuous extractor of the Kutscher Steudel type with the dimensions described by Krebs, Smyth & Evans (1940). The deproteinized filtrate is acidified with 0.1 vol of 50% (v/v) H_2SO_4 . The rate of extraction is illustrated by the following data. When 5.84 mg of α -ketoglutaric acid, dissolved in 15 ml of 2N H_2SO_4 were extracted, 71% appeared in the extract within 15 min, 90% within 30 min, 95% within 60 min. These figures depend of course on many factors, especially the design of the apparatus and the rate at which ether passes through the solution, it is therefore necessary to check the rate for each

set up. In order to have a safety margin we extract for 2 hr. The ether is then evaporated and the residue is dissolved in a few ml of water. It is usually divided into 2 parts, in one succinate is determined directly, whilst the other is acidified and treated with an excess of KMnO_4 as described by Krebs & Eggleston (1945).

This procedure eliminates interference by glutamic acid, glutamine and arginine, but if α -hydroxyglutarate is present the dinitrophenylhydrazone method (Krebs, 1938) remains the method of choice.

SUMMARY

1 Succinic dehydrogenase is inhibited by phospho 18 tungstate and phospho 24 tungstate, presumably because these reagents oxidize the SH groups of the enzyme.

2 Glutamic acid, whilst stable in the presence of potassium permanganate in pure solution, is oxidized by this reagent to succinic acid in the presence of certain deproteinized tissue extracts.

3 The above reactions may cause errors in the manometric determination of succinate and α keto glutarate. Procedures are described which avoid such errors.

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New Zealand Fish Oils

5 COMPOSITION OF THE FATS OF THE SCHOOL SHARK (*GALEORHINUS AUSTRALIS*, MACLEAY)

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The school or snapper shark (*Galeorhinus australis*) occurs in both New Zealand and Australian waters and, in common with related species found elsewhere, such as the North West Pacific soupfin shark (*G. zyopterus*) (Byers, 1940), the South American tolo (*G. mento*) (Pfister, 1936a, b) and the South African soupfin shark (*G. canis* Rond) (Molteno, Rapson, Roux, Schwartz & van Rensburg, 1945) is an im-

portant source of vitamin A rich liver oil (cf. Davies & Field, 1937, Jowett & Davies, 1938, Cunningham & Slater, 1939). Little, however, is known concerning the composition of oils from these species.

In most work on the fatty acid composition of fish oils from a given species, one sample only has been used so that there is no information as to the extent of the variation between individuals. This may be

large, as in New Zealand groper (*Polyprius oxy genctos*) liver oil (Shorland & Hilditch, 1938), or small as in New Zealand ling (*Genypterus blacodes*) liver oil (Shorland, 1939). In this work, therefore, the fats from eight specimens of school shark selected at random from one day's catch in Cook Strait on 16 February 1944 have been studied separately.

EXPERIMENTAL AND RESULTS

Information about the specimens used in this study is given in Table 1.

The sharks were divided by a transverse cut at the posterior gill slit into head, body, liver and rest of viscera. Each of the tissues was treated with twice its weight of 95% ethanol, held at the boiling point for 15 min and

Table 1 Details of school sharks used in this investigation

Sample (no)	Locality	Length (cm)	Weight (kg)	Weight of liver (g)	Sexual condition
1	Karori Rock (10 fathoms)	164	15.43	568	♀, commencing development
2	Panario patch (60-80 fathoms)	149	13.63	822	♂, undeveloped
3	" "	123	6.80	367	♀, undeveloped
4	" "	136	10.90	908	♀, undeveloped
5	" "	132	9.99	740	♀, undeveloped
6	Island Bay bank (60-80 fathoms)	151	24.95	1250	♂, commencing development
7	" "	144	12.27	568	♀, commencing development
8	" "	138	11.82	1080	♀, commencing development

Table 2 Proportions of tissues and distribution of fats in New Zealand school shark

Sample (no)	Head			Body			Viscera other than liver			Liver			oil in shark
	Oil		% total in fish	Oil		% total in fish	Oil		% total in fish	Oil		% total in fish	
	% total weight	(%)		% total weight	(%)		% total weight	(%)		% total weight	(%)		
1	21.3	0.2	3.3	67.7	0.5	20.5	7.3	0.6	3.4	3.7	23.1	66.8	1.3
2	21.6	0.2	1.1	66.1	0.5	8.7	6.3	1.0	1.6	6.0	55.9	88.6	3.8
3	21.6	0.3	3.2	66.3	0.5	16.3	6.7	0.6	2.0	5.4	29.6	78.5	2.0
4	17.7	0.1	0.4	69.0	0.5	7.2	5.0	0.7	0.7	8.3	52.7	91.7	4.8
5	18.2	0.4	1.5	69.3	0.7	9.9	5.1	1.2	1.3	7.4	57.6	87.3	4.9
6	10.7	0.1	0.3	78.4	0.4	10.6	5.9	0.8	1.6	5.0	51.8	87.5	3.0
7	23.2	0.2	2.3	66.2	0.3	9.8	6.0	0.9	2.6	4.6	37.6	85.3	2.0
8	19.7	0.2	0.7	65.7	0.5	5.5	5.5	0.9	0.8	9.1	60.7	93.0	5.8
South African soupfin shark	15.3*	0.48	3.5	78.1	0.71	26.8	1.7†	0.7	0.6	4.9	29.2	69.1	2.1

* Excluding foetuses. Recalculated from data given by Molteno *et al.* (1945).

† Intestine only.

Table 3 Characteristics of New Zealand school shark fats

Sample (no)	Liver				Body		Head
	Vitamin A*	Unsaponifiable matter (%)	Saponification equivalent	Iodine value (Wijs 1 hr)	Saponification equivalent	Iodine value (Wijs 1 hr)	Iodine value (Wijs 1 hr)
	$\left(\frac{E_{1\text{cm}}^{1\%}}{328\text{ m}\mu}\right)$						
	16 (%)						
1	14.6	31.4	410.0	185.0	366.5	125	112
2	3.6	8.1	317.1	178.0	346.5	146	140
3	0.6	4.5	309.5	183.9	340.5	154	118
4	0.1	2.5	306.5	185.9	328.0	136	103
5	0.4	4.0	307.7	176.8	326.5	136	109
6	2.2	6.4	319.6	170.7	354.0	153	116
7	1.9	8.9	321.0	149.0	301.5	119	137
8	0.6	3.3	308.0	171.7	327.0	127	129

* Spot tests with the Carr Price reagent showed that some of the body and visceral oils were somewhat richer in vitamin A than cod liver oil but no positive tests were given by the head oils.

then dried *in vacuo* at a temperature not exceeding 50°. The dried tissues were minced and extracted continuously with light petroleum, b.p. 50–70°, until on replacement with fresh solvent no further lipid was extracted. The final stages of extraction were facilitated by the addition of 5% absolute ethanol to the light petroleum, the ethanolic layer being subsequently withdrawn, evaporated *in vacuo*, re-extracted with light petroleum, and added to the main extract from which the petroleum was distilled, the last traces being removed *in vacuo* on the steam bath.

The proportions of tissues and distribution of fat in the fish are given in Table 2 and the characteristics of liver, body and head fat in Table 3.

As it was not feasible to complete the ester fractionation analyses of all the samples, a selection was made of the liver oils, while the head and body oils were combined to provide sufficient material for analysis. The head and body fats were further separated into phosphatide and glyceride fractions (cf. Shorland & Hilditch, 1938) yielding 63.6% phosphatide (P = 3.7%) and 36.4% glyceride (P = 0.7%). Qualitative tests showed that the liver oils contained only minor proportions of phosphatide, no separation was therefore made.

The fatty acid composition was determined by the method described by Hilditch (1941) and modified by Shorland & de la Mare (1944). For the purpose of indicating the course of the distillation of the methyl esters the results for school shark liver oil (no. 7) are set out in Table 4 as typical of the general results obtained. The component fatty acids of the fat are listed in Table 5.

DISCUSSION

Rapson, Schwartz & van Rensburg (1945) consider that the composition of the liver fats of marine teleostean fish is largely determined by the extent to which the liver is used as a site for fat storage. They found, in general, that the liver fats of fishes with diffuse oil systems had lower iodine values than the corresponding head, body or intestinal oils, while in the case of fishes with the fat storage localized in the liver the fat from this organ had an iodine value approximating to that of the corresponding body, head and intestinal oil. In the present work it is shown that in common with that of other elasmobranch fish the liver of the school shark is the main site of fat storage (cf. Table 2). Consideration of the iodine values of the liver fats (Table 3) shows that they exceed those of the head and body. Such comparisons of iodine values are complicated by the presence of unsaponifiable matter and by the fact that the head and body lipids are present to a considerable extent as phosphatides. With the methyl esters of the fatty acids, excluding unsaponifiable matter, as basis for comparison, liver oil samples nos. 1, 2, 6 and 7 showed iodine values of 137.3, 152.8, 152.4 and 134.7, respectively, as compared with values of 174.5 and 162.8 for the respective phosphatide and glyceride fractions of the combined head and body oils. The higher iodine value of the head and body oil fatty acids, as

Table 4. Fractionation of methyl esters of acids from liver fat of school shark no. 7

Methyl esters of 'liquid' acids (weight fractionated 39.5 g.)				Methyl esters of 'solid' acids (weight fractionated 11.7 g.)			
No.	(g.)	Saponification equivalent	Iodine value	No.	(g.)	Saponification equivalent	Iodine value
L ₁	1.305	246.8	40.1	S ₁	0.940	262.9	4.1
L ₂	2.929	270.8	78.9	S ₂	1.528	255.6	2.1
L ₃	3.894	291.8	92.8	S ₃	2.647	269.0	2.9
L ₄	2.496	295.4	95.7	S ₄	2.605	272.0	3.7
L ₅	6.549	296.6	99.1	S ₅	2.748	297.6	25.4
L ₆	4.087	315.0	168.8	S ₆	1.088	366.6	61.7
L ₇	2.970	322.9	169.6				
L ₈	2.953	334.4	257.2				
L ₉	2.502	341.6	299.4				
L ₁₀	3.241	344.0	308.8				
L ₁₁	1.607	347.2	273.7				
L ₁₂	1.653	359.3	212.6				
L ₁₃	2.971	365.9	183.4				
Total weight of fractions	39.157			Total weight of fractions	11.556		

'Liquid' esters excluding unsaponifiable matter

L₁₂ saponification equivalent 348.2, iodine value 204.0

L₁₃ saponification equivalent 359.1, iodine value 177.0

'Solid' esters excluding unsaponifiable matter

S₆ saponification equivalent 351.6, iodine value 79.2*

* The relatively high iodine value after resaponification is attributed to the removal of traces of residual phosphatides (cf. Hilditch & Shorland, 1937) which would tend to lower the iodine value in the original fraction.

Table 5 *Component fatty acids of the fats of the school shark*

Liver oil sample	(a) Weight percentages					Unsaturated acids					
	Saturated acids					C ₁₄	C ₁₆	C ₁₈	C ₂₀	C ₂₂	C ₂₄
1	13	17.1	6.5	1.5	—	0.6 (-2.0 H)	5.4 (-2.0 H)	25.4 (-2.6 H)	20.7 (-4.5 H)	21.5 (-7.6 H)	—
2	1.7	15.3	3.4	1.1	—	1.2 (-2.0 H)	5.9 (-2.0 H)	26.6 (-2.4 H)	19.5 (-5.8 H)	25.3 (-6.6 H)	—
6	2.4	15.2	3.0	—	—	1.1 (-2.0 H)	6.2 (-2.0 H)	31.7 (-2.4 H)	20.2 (-5.4 H)	19.6 (-8.8 H)	—
7	3.9	16.7	5.3	0.1	1.0	0.7 (-2.0 H)	5.3 (-2.0 H)	20.5 (-2.3 H)	15.5 (-4.5 H)	22.8 (-6.5 H)	2.2 (-4.0 H)
Head and body glycerides	0.2	7.8	19.0	5.2	3.4	0.2 (-2.0 H)	5.2 (-2.0 H)	13.5 (-2.2 H)	22.8 (-6.1 H)	22.7 (-12.0 H)	—
Head and body phosphatides	0.3	9.9	13.3	0.3	1.1	1.1 (-2.0 H)	4.3 (-2.0 H)	22.8 (-2.3 H)	16.1 (-6.5 H)	30.8 (-10.0 H)	—
	(b) Molecular percentages										
	C ₁₄	C ₁₆	C ₁₈	C ₂₀	C ₂₂	C ₂₄					
1	1.7	19.4	6.6	1.4	—	0.7 (-2.0 H)	6.2 (-2.0 H)	26.3 (-2.6 H)	19.6 (-4.5 H)	18.1 (-7.6 H)	—
2	2.2	17.3	3.5	1.0	—	1.5 (-2.0 H)	6.7 (-2.0 H)	27.3 (-2.4 H)	18.4 (-5.8 H)	22.1 (-9.6 H)	—
6	3.1	17.0	3.7	—	—	1.4 (-2.0 H)	6.9 (-2.0 H)	32.2 (-2.4 H)	18.8 (-5.4 H)	16.9 (-8.8 H)	—
7	4.9	18.8	5.4	0.1	0.8	0.9 (-2.0 H)	6.0 (-2.0 H)	27.1 (-2.3 H)	14.5 (-4.5 H)	10.7 (-6.5 H)	1.8 (-4.0 H)
Head and body glycerides	0.3	9.0	19.8	4.9	3.0	0.2 (-2.0 H)	6.0 (-2.0 H)	14.2 (-2.2 H)	22.1 (-6.1 H)	20.5 (-12.0 H)	—
Head and body phosphatides	0.5	11.4	13.7	0.3	1.0	1.4 (-2.0 H)	5.0 (-2.0 H)	23.8 (-2.3 H)	15.5 (-6.5 H)	27.4 (-10.0 H)	—

The quantities in brackets are the values of the mean unsaturation, thus (-4.0 H) indicates an average unsaturation corresponding with two double bonds, but does not necessarily imply the presence only of a diethylenic acid

Table 6 *Fatty acid composition of school shark liver oil no 2 before and after hydrogenation*

	Total groups of acids (mol. %)					
	C ₁₄	C ₁₆	C ₁₈	C ₂₀	C ₂₂	C ₂₄
Before hydrogenation	3.7	24.0	30.8	19.4	22.1	—
After hydrogenation	2.8	24.3	33.4	16.7	22.5	0.3
Difference	+0.9	-0.3	-2.6	+2.7	-0.4	-0.3

compared with those of the liver, is in agreement with the views put forward by Rapson *et al* (1945)

The variation in fatty acid composition between the liver oils (cf Table 5) reaches a maximum difference in the C₁₈ unsaturated acids, where the difference between samples 6 and 1 amounts to 6.3 units weight %. Other similar differences are shown in the C₂₀ unsaturated acids, sample 1 containing 5.2 units % more than sample 7, while in the case of the C₂₂ unsaturated acids sample 2 is shown to contain 5.7 units % more than sample 6. In assessing differences of this order it is relevant to consider the accuracy of the ester fractionation method.

Comparison of the original oil with the fully hydrogenated sample (Table 6), shows that by this method in two consecutive analyses differences of

up to 2.7 units % may be expected. Using similar comparisons, Hilditch & Houlbrooke (1928), Shorland (1939) and van Rensburg, Rapson & Schwartz (1945) found respectively, differences of up to 1.7, 1.7 and 2.5 units %. Similarly Lovern (1942) found, in duplicate analyses of cod liver oil, differences between the same fatty acid groups of up to 1.8 units %. By far the most exhaustive and comprehensive analyses carried out on oils, hydrogenated to different iodine values and prepared from the same sample, are those by Hilditch & Terleski (1937) on whale oil and by Harper & Hilditch (1937) on cod-liver oil.

As claimed by these workers (Harper, Hilditch & Terleski, 1937) the final results for the component fatty acids are not necessarily accurate to more than 2 or 3 units % in the case of the higher complex

unsaturated acids Our interpretation of their claim is that this degree of accuracy has reference to the extent of the deviation from the mean value, so that, when considered from the point of view of the differences likely to be encountered between similar homologous groups of acids in two consecutive analyses, the expected differences are of the order of up to 6 units % Using the results of the above investigators, the standard deviations of each homologous group of acids have been calculated (Table 7)

Table 7 *Standard deviation of ester fractionation analyses calculated from the data of Hilditch & Terleski (1937) and of Harper & Hilditch (1937)*

Standard deviation from mean of homologous groups of acids (mol %)

	C ₁₄	C ₁₆	C ₁₈	C ₂₀	C ₂₂	C ₂₄
Whale oil (6 analyses)	±0.83	±1.54	±1.65	±1.14	±0.75	—
Cod liver oil (8 analyses)	±0.62	±1.07	±1.04	±1.81	±1.19	±0.39

It is generally considered that, for the results of a single analysis on two different oils to be significant, their difference, for a first approximation, should differ by more than three times the standard deviation of a single observation. In the present series of analyses, because of the relatively small weight of sample used (c. 50 g), it was not expected that the accuracy would be greater than that obtained in the case of the cod liver oil and whale oil analyses, where larger weights were taken and the fractionation more detailed. However, because of the use in the present work of a more efficient fractionating column as described by Longenecker (1937), the relatively small weight of oil taken for analysis is compensated to some extent by the relatively greater degree of separation of the esters, as compared with that obtained with the Willstatter bulb used in the cod liver oil and whale oil analyses. It thus seems that the larger differences in the component fatty acids of the school shark liver oils referred to above are possibly significant, but no conclusions regarding the smaller differences appear to be justified.

Comparing the fatty acid composition of the head and body lipids with that of the liver oil (Table 5), it will be seen that the former have appreciably less C₁₈ unsaturated acids and palmitic acid but more stearic acid. Attention should also be drawn to the high mean unsaturation of the C₂₀ and C₂₂ unsaturated acids of the head and body lipids as compared with those of the liver. The differences normally observed between the fatty acid composition of corresponding phosphatides and glycerides (cf. Hilditch & Shorland, 1937), including increased proportions of stearic acid, and C₂₀ and C₂₂ unsaturated acids in the former as compared with the latter, are not shown in this work.

van Rensburg *et al.* (1945) extended their earlier view that in fishes with diffuse systems of fat storage

there is a direct relationship between the iodine values of the head and body oils and the oil contents of these organs, and put forward the hypothesis that these variations correspond to preferential deposition and utilization of C₂₀ and C₂₂ unsaturated acids, as well as changes in the degree of unsaturation of the acids when the fish are in fat and thin condition respectively. They suggested further that the same may be true of the liver oils of fish where, as in the present case, the liver is the main site of fat storage.

In this connexion they showed that, by comparing the fatty acid composition of the liver oils of *Merluccius merluccius* (50% oil in liver) with that of *M. capensis* (28% oil in liver) and *M. gayi* (23% oil in liver), there was a decrease in the amount of C₂₀ and C₂₂ unsaturated acids from 45.0 to 31.7% with decreasing oil content.

In the present work the oil content of the liver of the school shark has been shown to vary (Table 2) from 23.1% (sample 1) to 55.9% (sample 2), without any marked effect on the fatty acid composition, suggesting that in the school shark it is not a major factor in determining fatty acid composition. It may also be shown (Table 8) that, in other fish where the liver is the main fat depot and the distribution and amount of oil are similar to that of the school shark, the fatty acid composition differs considerably from that of the latter.

The New Zealand ling and school shark referred to in Table 8 have been taken from similar localities in Cook Strait, but the former is distinguished from the latter by containing highly significantly more C₁₈ and C₂₀ unsaturated acids and highly significantly less C₂₂ unsaturated acids in the liver oil. Thus, although the mode and extent of fat storage in the liver have been shown (cf. Rapson *et al.* 1945) to be associated with wide differences in fatty acid composition, for such fish as the groper and the *Merluccius* species (van Rensburg *et al.* 1945) these factors have not been found important in the school shark. The present comparison of the New Zealand school shark with the similar if not identical Australian species, and with the ling, suggests that in some cases the species factor is of primary importance in determining the fatty acid composition of the liver oil.

It is quite possible that the factors which determine the fatty acid composition of the liver oils of

Table 8 Comparison between the fatty acid composition of the liver oil of the school shark and of New Zealand ling*

Fatty acids (% w/w)													Liver oil expressed as percentage of total oil in fish	Oil in fish (%)
Saturated					Unsaturated									
C ₁₄	C ₁₆	C ₁₈	C ₂₀	C ₂₂	C ₁₄	C ₁₆	C ₁₈	C ₂₀	C ₂₂	C ₂₄				
New Zealand school shark	2.3	16.1	4.7	0.7	0.2	0.9 (-2.0 H)	5.7 (-2.0 H)	27.6 (-2.3 to -2.6 H)	19.0 (-4.5 to -5.8 H)	22.3 (-0.5 to -9.6 H)	0.5 (-4.0 H)		69-89	1.3-3.8
Mean value														
Standard error (4 samples)	±0.55	±0.45	±0.75	±0.40	±0.25	±0.15	±0.20	±1.4	±1.1	±1.3	±0.55			
Australian school shark	2.6	18.7	5.1	0.1	—	0.3 (-2.0 H)	8.4 (-2.0 H)	27.2 (-2.4 H)	17.8 (-6.0 H)	19.8 (-12.0 H)	—			
Mean value													c 90	2.9
New Zealand ling (Shorland, 1939)	1.8	16.4	2.5	—	—	—	7.2 (-2.0 H)	36.1 (-2.1 to -2.6 H)	24.3 (-4.9 to -5.7 H)	11.7 (-0.9 to -9.7 H)	—			
Standard error (7 samples)	±0.17	±0.30	±0.31	—	—	—	±0.47	±0.64	±0.50	±0.85				
* Shorland (1939)														

* Shorland (1939)

elasmobranchii differ considerably from those operating in teleostean species. As shown by Hilditch (1941) the former have a much greater range of variation in the nature of the unsaturated acids, which vary from predominantly monoethenoid types to types which are even more unsaturated than those normally encountered in teleostean species. These facts, together with other remarkable differences in the amount and nature of the unsaponifiable matter in the liver oils of elasmobranchii, tend further to emphasize the importance of the species factor in determining the fatty acid composition of this group. Lovern (1942) has classified elasmobranch liver oils into four main groups on the basis of the nature and amount of unsaponifiable matter and the fatty acid composition. Consideration of the school shark liver oil shows that the higher unsaturated acids are largely polyethenoid, and the fatty acid composition is not very different from that of the average marine fish oil. In this respect the school shark is shown to conform to Lovern's first group from which, however, it must be distinguished by the presence of much more unsaponifiable matter (2.5-31.4%) as compared with usually not more than 1 or 2% considered typical of that group.

SUMMARY

1 Studies of the distribution of oil in eight specimens of school shark (*Galeorhinus australis*) showed that the liver, which varied in oil content from 23.1 to 60.7%, contained from 66.8 to 93.0% of the total oil reserves of the fish. Ester fractionation analyses of four of the liver oils, and of the phosphatide and glyceride fractions of the combined head and body lipids, showed that the liver fatty acids contained a higher content of palmitic acid and of C₁₈ unsaturated acids, but less stearic acid than the head and body lipids.

2 In regard to most groups of fatty acids the liver oils showed great similarity. The wider differences in content of C₁₈, C₂₀ and C₂₂ unsaturated acids amounting to as much as 6.3 units % are thought to lie outside the experimental error, but variations in accuracy shown by the results of various investigators are such as not to preclude such differences being due to experimental error.

3 Consideration of the variations in the oil content as a factor in determining the fatty acid composition of *G. australis* liver oil suggest that this fish is not influenced appreciably in this way, and thereby presents a striking contrast to the teleostean species studied by Rapson and collaborators.

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Studies on Suramin (Antrypol, Bayer 205)

6 FURTHER OBSERVATIONS ON THE DETERMINATION OF SURAMIN IN WHOLE BLOOD AND SERUM

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The amount of the trypanocidal drug suramin present in the blood serum or plasma of man, and any other animal which has received an injection of the drug, can readily be determined by a method previously described (Dangerfield, Gaunt & Wormall, 1938, Boursnell, Dangerfield & Wormall, 1939). The technique of this determination has deliberately been made as simple as possible in order that the method might be of use in tropical countries, even in regions where the simplest laboratory facilities are not available. The possibility has always been kept in mind that medical officers in regions well removed from the central hospitals might require blood suramin determinations on some of their treated trypanosomiasis patients. The drug has a very definite prophylactic value, and plasma suramin determinations might be made in future on large groups of people who have been injected with the drug before being exposed to infection.

Serum and plasma from suramin-containing blood contain the same concentration of suramin, and either material serves for the determination of the amount of the drug in the blood. Should the serum or plasma become grossly infected, however, the resulting bacterial changes might produce amines and other compounds which would vitiate the results. Experiments have, therefore, been carried out to determine whether any simple antiseptic can be added to the serum or plasma to preserve it for subsequent suramin determinations without interfering with the colorimetric determination. Experiments have also been carried out to investigate the reliability of suramin determinations on whole blood samples. Although these investigations were primarily designed for the above mentioned purposes, they have also furnished useful information about the distribution of the blood suramin between the cells and the plasma.

METHODS

Suramin determinations These were carried out as described previously (Dangerfield *et al* 1938, Boursnell *et al* 1939) The volume of each sample was made up to 3.0 ml with distilled water before the acid hydrolysis. Blank determinations were made on blood serum or plasma to which no suramin had been added, and these control solutions were placed in the 'backing' tube of the comparator to compensate for a small amount of 'non suramin' amine precursor in blood samples.

Some commercial samples of suramin when hydrolyzed, diazotized, and coupled with methyl α naphthylamine, gave a slightly stronger pink colour than did the sample used originally for the preparation of the Tintometer discs, i.e. they gave a 'recovery' of 101-108%, but this difficulty was readily overcome by standardizing each fresh batch of suramin. These slight differences between different suramin preparations might be partly due to differences in their water content (see also Hawking, 1941). Since, however, all our samples were dried *in vacuo* over CaCl_2 , it seems more probable that there is a slight variation in the composition of commercial suramin.

RESULTS

The recovery of suramin added to whole blood

Varying amounts of suramin were added to defibrinated or oxalated blood (ox, rabbit and human) and determinations were carried out on the whole blood, and in some cases on the separated cells and plasma. The results of typical experiments (Table 1) show that the recovery of the added drug is low and very variable. Duplicate determinations usually gave good agreement, but the percentage recovery

with any one sample of blood varied with the amount of suramin added. The latter observation excludes the use of a 'recovery' factor for calculating the true suramin content of whole blood.

It was thought possible that the recovery of suramin might be improved if the acid hydrolysis of the whole blood sample were more vigorous, e.g. by the use of a larger volume of HCl or by hydrolyzing for 9 hr instead of the customary 6 hr. Where 5 ml of HCl were used, the recovery (69-97%) was higher than that obtained with 3 ml, but the difference was not sufficiently marked to justify this change of technique. An increase in the duration of acid hydrolysis led to slight or negligible increases in the recovery of suramin added to whole blood, and more prolonged acid hydrolysis is not desirable since it has been found that further heating beyond the normal 6 hr period leads to loss of the hydrolyzed suramin (Dangerfield *et al* 1938).

The recovery of suramin added to washed red blood cell suspensions Since there is approximately complete recovery of suramin added to serum or plasma, it was concluded that the lower recovery with whole blood samples was due to constituents of the red cells, or more probably to products produced by acid hydrolysis of these cells. Suramin determinations have therefore been made on mixtures of suramin and suspensions of washed red blood cells (ox and human), again the recovery was very low.

Possible reasons for the low recovery of suramin added to whole blood The low recovery of suramin from whole blood samples is apparently due to some constituent of the red cell or a product formed by

Table 1 Recovery of suramin added to whole blood

(Suramin was dissolved in 0.9% (w/v) NaCl and 1.0 ml. of the solution was added to fresh defibrinated ox or human blood or oxalated rabbit blood.)

Type of blood	Volume of blood (ml)	Suramin added (mg)	Acid hydrolysis		Recovery* (%)
			Amount of 10.3N HCl (ml.)	Duration (hr)	
Ox	0.50	0.44	3.0	6	86
			3.0	9	86
			5.0†	6	97
	2.0	0.44	3.0	6	69
			3.0	9	75
			5.0†	6	83
Rabbit	2.0	0.50	3.0	6	52
			5.0†	6	69
	2.0	2.0	3.0	6	60
			5.0†	6	76
Human	2.0	1.03	3.0	6	66
			5.0†	6	76

* In this and subsequent tables the results recorded are average values for two duplicate determinations.

† When 5.0 ml of 10.3N HCl were used, the hydrolyzed solution was diluted with distilled water to 10.7 ml (as against 10.0 ml. when 3.0 ml. of HCl were used) to give the right acidity (approx. 3N) for satisfactory diazotization and coupling.

hydrolysis of the cells, which inhibits the diazotization or coupling, or which reacts with the amines derived from suramin. Experiments were therefore carried out to determine the recovery of suramin after its addition to HCl-hydrolyzed blood or red blood cells. The results showed that quantitative recovery was obtained under these conditions. The loss appears to occur, therefore, when the suramin is heated with HCl in the presence of red cells.

The preservation of serum for suramin determinations

Many experiments have been carried out with a variety of commonly used antiseptics to determine which are satisfactory for the preservation of serum for subsequent suramin determinations. The following antiseptics were added to serum from rabbits injected with suramin, or to normal human serum to which suramin had been added: ether, chloroform

Table 2 *Distribution of suramin added to whole blood*

Mixture	Defibrinated ox blood (ml)	Suramin added in 10 ml of 0.9% NaCl (mg)	Suramin found (mg/21 ml of the mixture, or fraction obtained from 21 ml. of the mixture)		
			Whole blood	Cells*	Plasma
A	20	20.0	12.8	1.4	17.0
B	20	3.5	2.4	0.2	3.2

* For reasons given in the text the cells were not washed, and the small amount of suramin found in them was probably present in the plasma adhering to the cells.

Distribution of added suramin between the red cells and plasma of whole blood

As a preliminary to an investigation on the distribution of suramin between the plasma and cells of the blood of animals injected with the drug, experiments have been carried out to determine how suramin added to the whole blood is distributed. Suramin (dissolved in a little 0.9% NaCl) was added to fresh defibrinated ox blood, and samples of the well shaken mixture were centrifuged and the plasma and red cells separated as completely as possible. The cells were not washed in this experiment, in order to avoid the loss of any suramin which might be loosely combined with the cell membrane, or any which might diffuse through the cell wall during the washing. The results (Table 2) show that the added suramin became associated completely or almost completely with the plasma. The small amount present in the red cell fraction in this experiment can almost certainly be accounted for by the small amount of plasma remaining in the unwashed red cell fraction.

The recovery of the suramin from the whole blood mixtures (64% for A and 69% for B) was similar to that obtained in other comparable experiments. The total recovery from the separated plasma and red cells, i.e. the sum of the values for the two fractions, was quite satisfactory (92 and 97%), the slight loss being due to incomplete recovery of the small amount of the drug present in the red cell fractions.

In other experiments, suramin was added to suspensions of red blood cells to determine whether any of the drug became attached to the cells either immediately or when the mixtures were allowed to stand, with gentle shaking, for 3 hr. The results of these experiments, some of which are given in Table 3, show that no significant amount of the added drug became attached to the red cells

and toluene (0.3, 0.2 and 0.2 ml respectively/2 ml of serum), phenol and merthiolate (to give concentrations of 0.2–0.4 and 0.018% respectively), 10 N-HCl (3 ml/2 ml serum). Suramin determinations made on these treated sera after they had been standing at 17–25° for periods of 1–5 weeks showed that ether,

Table 3 *Addition of suramin to suspensions of washed red blood cells*

(Fresh defibrinated blood was centrifuged and the red cells washed several times with 0.9% NaCl solution, and then suspended in sufficient 0.9% NaCl to give a volume equal to that of the original blood sample.)

Mixture A - 20.0 ml of ox red blood cell suspension + 22.1 mg of suramin in 1 ml of 0.9% NaCl

Mixtures B and C - 20.0 ml of human red blood cell suspension + 21.6 mg of suramin in 1 ml of 0.9% NaCl

The mixtures were kept at 21° for 15 min (A and B) or 3 hr (C) with occasional gentle shaking, and 5 ml samples of each were then centrifuged. The cells were washed three times with 0.9% NaCl (3 ml each time), and the washed cells suspended in 0.9% NaCl to give a final vol of 5.0 ml. The supernatant solution and washings from each sample were combined, and 0.9% NaCl was added to give 20.0 ml.)

Mixture	Suramin added (mg)	Suramin found (mg/21 ml of A, B or C, or fraction obtained from 21 ml)		
		Original cell suspension	Washed red cells	Super- natant plus washings
A	22.1	17.0	0.0	22.1
B	21.6	15.8	0.2	21.0
C	21.6	16.4	0.2	20.8

chloroform and toluene are not completely satisfactory for preservation, the results usually agreed with determinations made before the preservation of the serum, but occasionally low results were obtained. The HCl treated samples were sometimes

satisfactory, as was noted by Hawking (1940 a), but they occasionally gave surprisingly low results, no explanations for this can be offered at present. The results with the sera preserved with phenol or merthiolate were invariably satisfactory. Serum samples to which no antiseptic had been added became grossly infected when kept at room temperature for 1-5 weeks, and they often gave high suramin values.

DISCUSSION

Determinations of the amount of suramin in the blood can yield useful information about the prophylactic value of the drug, and about the blood level which might reasonably be expected to confer immunity against trypanosomiasis. Vierthaler & Boselli (1939), for example, found that a concentration of 1.3 mg of the drug/100 ml of plasma, as determined by the method developed in our laboratory (Boursnell *et al* 1939, Dangerfield *et al* 1938), was sufficient to protect rabbits against infection when injected with *Trypanosoma brucei*. Hawking (1940 a, b) concluded that defective accumulation of the drug in the blood was one of the main factors responsible for relapses in three patients treated with suramin for African sleeping sickness; he also found that numerous living trypanosomes reappeared in the blood of one patient even though the plasma contained 4.5 mg of suramin/100 ml.

It is possible that investigators in tropical countries might wish to make suramin determinations, by our method, on samples of whole blood, but the low results obtained in our experiments indicate that plasma or serum must be used to obtain a true picture of the blood suramin level. Where samples of sera or plasma have to be kept for some considerable time before suramin determinations can be carried out, it is advisable that some antiseptic should be added, gross infection sometimes produces appreciable amounts of amines, or amine precursors, which cause a high result to be obtained in the suramin determination. Of the limited number of antiseptics tested by us, phenol

and merthiolate have proved most satisfactory, and it is recommended that 0.2 ml of 5% (w/v) phenol or 0.1 ml of 0.5% (w/v) merthiolate should be added to 2 or 3 ml of plasma or serum. The use of phenol for this purpose might be specially recommended, since it has been shown (Wormall, 1933) that phenol-treated serum can be used for the diagnostic red cell adhesion test for human trypanosomiasis (Duke & Wallace, 1930, Wallace & Wormall, 1931).

The long retention of injected suramin in the animal body can be attributed to the combination of the drug with the plasma and tissue proteins. In view of the ready combination of the drug with many proteins (for the literature see Boursnell & Wormall, 1939, Dewey & Wormall, 1946, Spinks, 1948), it seems rather surprising that no significant amount of suramin combines with the red blood cells. In our experiments, red blood cells took up no suramin when they were mixed with suramin solutions and left for up to 3 hr, and, in experiments which will be reported later, little suramin has been found in the red cells of the blood of rabbits injected with the drug.

SUMMARY

- 1 Suramin determinations made on whole blood samples, by the method previously found satisfactory for serum and plasma, show a low recovery of the suramin.

- 2 This low recovery is due to constituents of the blood cells.

- 3 Suramin added to whole blood is almost completely confined to the plasma, and none combines with the red cells.

- 4 The addition of phenol or merthiolate to serum samples is recommended if these samples have to be kept for some time before suramin determinations are made.

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The Fractionation of Potato Starch

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The fractionation of starch by precipitation with butanol and similar alcohols, first developed by Schoch (1942), stimulated research in this field by providing the first efficient method of preparing in reasonably pure form the components, amylose and amylopectin. These names have often been applied to the corresponding fractions obtained by earlier methods in various degrees of purity, and in consequence are associated in the literature with many conflicting statements. They are nevertheless retained here, it being understood that amylose, consisting essentially, if not solely, of unbranched chains of α -glucopyranose units, is the fraction precipitated from starch paste by butanol, amylopectin remaining in dispersion.

It is supposed that amylose is completely hydrolyzed to maltose by β amylase, although the evidence for this is not entirely satisfactory, since pure substrate and enzyme have rarely, if ever, been employed in the same experiment. Tests of the purity of starch fractions include the limit of β amylase action, analysis of iodine coloration and potentiometric titration with iodine, the first two have been extensively employed in this work. In the present investigation, attempts have been made to obtain pure amylose and amylopectin by the known precipitation methods, and if possible to improve upon these methods.

Schoch (1942) autoclaved starch paste with butanol or isoamyl alcohol, and later (Schoch, 1945), after autoclaving alone, added excess of pentasol, a commercial mixture of primary amyl alcohols. The liquid was cooled very slowly, amylose coming out in crystalline needles or rosettes which were separated by high speed centrifugation. It was purified by recrystallization from the same solvent mixture. Amylopectin was precipitated from the mother liquor with methanol. Autoclaving is considered by Schoch (1945) to be essential to the success of the fractionation, otherwise, the fractions are markedly contaminated with one another.

Haworth, Peat & Sagrott (1946) precipitated the amylose from non autoclaved starch paste in the cold with thymol. The avoidance of autoclaving is a valuable feature of this method, since mild hydrolytic decomposition of amylose and amylopectin, even in 2 hr. at 120° at pH 6 as employed by Schoch, may be feared. These authors also suggested the use of the cyclohexanols as precipitants, and Whistler & Hulbert (1945) that of the nitroparaffins.

EXPERIMENTAL

Materials Commercial samples of potato starch, the best obtainable, were employed. One sample gave a paste of pH 4.5. By washing the starch three times with 0.001N K_2CO_3 , followed by distilled water, the reaction of the paste was changed to pH 5.8–6 as required by Schoch (1942). This starch yielded about 17% of purified amylose. Another sample, for example, yielded 20% amylose and a less viscous starch paste.

Starch was mixed to a cream with cold water, and added to boiling water with vigorous stirring. After gentle boiling for 30 min. to yield a homogeneous paste, the latter was either (1) treated with 0.05% NaCl and rapidly cooled, or (2) heated in an autoclave at 18–20 lb. pressure for 2 hr. as described by Schoch, the NaCl being omitted. The pastes contained about 1.5% of starch.

Determinations of yields of fractions A weighed sample of a solution was evaporated to dryness, dried at 104° and allowance made for any non carbohydrate constituent if present.

Determination of blue value The procedure of McCready & Hassid (1943) was followed with slight modification, but different units were employed. A solution containing 1 mg. of carbohydrate/100 ml. and 0.002% of I_2 in 0.02% KI was read in a 1 cm. cell in a Spekker absorptiometer against iodine, using (unless otherwise stated) the 608 filter (6500–7000 Å transmission), which corresponds closely to that employed by McCready & Hassid. The reading under these conditions is the blue value. In some investigations, other filters, e.g. 607 (5900–6300 Å), were also employed (see text).

Fractionation procedure

Preliminary experiments yielded 23% of crude and 17% of fully purified amylose from one potato starch by Schoch's (1942) method, using butanol. Purification was achieved by redissolving the crude amylose to 0.7–1.0% concentration in hot water saturated with butanol, and cooling from 80° or 90° over a period of 3–5 hr. The precipitate was washed with butanol saturated water until the washings no longer gave any colour reaction with I_2 , redissolved at 80°, reprecipitated and washed. Three reprecipitations, involving nine washings, yielded a product from which no more amylopectin could be extracted, as indicated by the absence of iodine coloration or dissolved carbohydrate in the mother liquor.

Amylose precipitated by thymol (Haworth *et al.* 1946), or cyclohexanol followed by thymol, was separated as soon as possible by centrifuging, redissolved in hot butanol saturated water, reprecipitated and washed several times as described above.

The original mother liquor was concentrated *in vacuo* and the amylopectin precipitated with ethanol. Foaming created difficulties when thymol had been used, and the evaporation was sometimes omitted.

The best amylopectin was obtained as follows. To 1.5–2% starch paste (prepared with NaCl as in (1) above) cyclohexanol was added, drop by drop with stirring, up to 0.15–0.2% of the paste. Stirring must not be continued until the paste becomes milky. After standing 3 days, 0.15% of powdered thymol was brought into suspension by gentle stirring. The amylose precipitated within 2 days and was separated by centrifuging. The amylopectin had a blue value of about 0.02, whereas that from the thymol precipitation alone was about 0.025–0.03. If stirring with cyclohexanol increases the opalescence of the paste the resulting amylopectin is found to have an increased blue value.

Precipitation of amylose from starch paste by the method of Haworth *et al.* (1946), followed by rapid drying, gave a product which was only slightly soluble in water. The original centrifuged preparation could, however, be purified by recrystallization from butanol saturated water, which was preferred to thymol water for this purpose. In effect, it was thereby converted into the more stable butanol complex, and it was usually stored in this form without drying.

There appears to be no necessity to heat the starch paste under pressure before precipitating the amylose, even if butanol is used. Two preparations of amylose were carried out by butanol precipitation, the one from paste autoclaved for 2 hr. at 120°, the other from paste which never rose above 90° at any time. The latter gave some trouble, but the redissolved amylose was successfully separated from remnants of burst starch granules, which could not be easily removed from the original paste. The two amylose preparations were readily soluble, and gave similar blue values (0.33), but that from autoclaved paste was hydrolyzed by β amylase much the faster, each reaching the same limit. The corresponding amylopectins possessed identical blue values (0.024), and hydrolyzed at almost the same rates, the 120° preparation only slightly the faster.

So far as the amylose was concerned it did not greatly matter which precipitant was used, butanol, thymol or cyclohexanol followed by thymol, provided that the separated complex was promptly washed and recrystallized from butanol water. In one instance, using the same non autoclaved starch paste, butanol and thymol precipitations were performed side by side. The butanol amylose recrystallized three times, gave a 17.2% yield, blue value (n_D) 0.355. Thymol precipitated amylose, recrystallized three times from butanol water, gave a 17.7% yield, n_D 0.345. The light absorptions of the respective blue liquids were almost identical from 4300 to 5800 Å. However, the thymol preparation gave the lower absorption from 5800 to 7000 Å. Under the same conditions, both amyloses hydrolyzed at the same rate with β amylase, and reached the limit of 99.5% of theoretically obtainable maltose.

The crude thymol amylose was bulky and did not adhere to the walls of the vessel but after recrystallization from butanol water it occupied only 40% as much space as before and adhered to the walls. It underwent no further change in these respects during further recrystallizations from butanol water. It was found unsatisfactory to leave it long in contact with thymol water as it lost solubility and affinity for I_2 .

The butanol amylose complex, on heating with water saturated with butanol begins to disperse at 65° to form an

opalescent solution which clears at about 73°. On cooling slowly the phenomenon of supercooling may be observed as far down as 35°.

Schoch (1942, 1945) stipulated that the reaction of potato starch paste must be about pH 5.0–6.3 for successful fractionation. In this work, there were indications that wider variations in pH were permissible, indeed, successful precipitations from non autoclaved paste were effected even at pH 4.1 and 9. But certain differences in behaviour were noted in extreme cases. Thus, amylose precipitated from non autoclaved paste by butanol, thymol or cyclohexanol followed by thymol, brought by HCl addition to pH 4.1, was more easily washed and freed from amylopectin than in parallel precipitations at pH 6 and 9. It also showed a greater tendency to retrograde in neutral, hot aqueous solution, and when dried on a watch glass it was opaque. It was hydrolyzed by β amylase in dilute solution faster than amylose precipitated at pH 6 or 9 hydrolyzed under identical conditions.

No significant differences were noted in the amylopectins except that the lowest blue values were obtained in the cyclohexanol + thymol method (Table 2).

RESULTS

General properties of amylose

Amylose forms a viscous solution when concentrated, which may cool to a gelatinous consistency. Solutions of over 0.5% retrograde fairly readily, and this has been a source of difficulty in previous work. Whenever amylose approaching a high degree of purity has been prepared in these laboratories, e.g. by electrophoresis, retrogradation has been liable to occur, rendering the product permanently insoluble in water and useless for many purposes. Precipitates from hot solutions on cooling may not be retrograded material, but may redissolve with more hot water. Retrogradation may be avoided by high temperature, low concentration, maintenance of neutral pH, and over long periods by preservation in the form of a complex with butanol, thymol and nitroparaffins. Recrystallization from butanol water need not involve a cooling period greater than 3–5 hr. In the form of the butanol complex, i.e. in the presence of butanol saturated water, even the purest amylose is stable for months and shows no signs of retrogradation. The supernatant liquid in such a case contains less than 1 mg solid matter/100 ml and gives only a yellow colour with iodine.

Criteria of purity of amylose

The distinction between the iodine colorations of amylose and amylopectin consists essentially in the intensity of the blue. While amylopectin shows no great variation from 5000 to 8000 Å, amylose exhibits a marked minimum transmission near 6000 Å. Kerr (1944) places this at 6000–6200 Å for potato amylose which corresponds to light filter 607 as

used with the Spekker absorptiometer. For different preparations we have found from 5800 to 6100 Å, the highest reading on the absorptiometer being obtained with the 607 filter. After making allowance for variation in experimental error when using these filters, the one should be chosen which gives the greatest numerical difference between amylose and amylopectin. This is when the need is to characterize different preparations of amylose or to measure amylose present as an impurity in amylopectin. For the reverse purpose, that of measuring

blue value of both the amylopectin which remains contaminated with it and the amylose which has lost it. A perfectly representative sample of amylose cannot be obtained unless the first precipitation is performed in the right way.

The blue value was found to be unaffected by small quantities of butanol and maltose. It is chiefly influenced by the temperature at the time of reading, and by the conditions under which the amylose has been crystallized from solution, and, generally, the amount of exposure it has had to hot

Table 1 *Blue values of amylose after various periods of heating and cooling*

	Light filter (no.)	Blue values			
		Original sample	Original sample dissolved, heated, saturated with butanol and cooled		
			From 70° in 3.5 hr	From 90° in 3.5 hr	From 90° in 1.5 hr
Solution in cold water	608	0.348	0.318	0.336	0.352
	607	0.361	0.335	0.350	0.369
	606	0.292	0.285	0.290	0.298
Solution held at 90° for 10 min before testing	608	0.339	0.316	0.330	0.350
	607	0.356	0.335	0.346	0.364
	606	0.291	0.277	0.287	0.296
Solution held at 90° for 30 min before testing	608	—	0.302	0.330	0.316
	607	—	0.332	0.348	0.335
	606	—	0.272	0.287	0.277

Filter 608, 6500–7000 Å., 607, 5900–6300 Å., 606, 5700–5900 Å.

amylopectin or red iodine complexes in amylose, the filter selected should transmit light of about 4800–5000 Å., the 603 filter being indicated.

Failure to remove amylopectin or other carbohydrate by recrystallization from cooling butanol-water solution is an indispensable criterion of purity. The process should be accompanied by no change in blue value (so long as recrystallization is brought about under the same conditions and is complete). Short of adequate investigation of molecular structure, perhaps the best further criterion available to us is the completeness of hydrolysis by pure β amylase together with some kinetic features of this hydrolysis which are dealt with in a separate communication.

The blue value is of use during the process of freeing amylose from adherent amylopectin, and its value rises to a maximum during successive recrystallizations. But the value may be different in one preparation from that of another and seems to depend both on the initial conditions and on those of the final recrystallization.

Some amylose is normally left behind in the amylopectin, probably relatively low molecular amylose, which is difficult to remove. That fraction of the native amylose which is not precipitated in the first instance will vary from process to process, and from precipitant to precipitant, and will influence the

water. Table 1 shows a series of values (measured with three filters) of one specimen dissolved and recrystallized under different conditions. The products (butanol complex) were each dissolved in three ways. These results mainly serve to show that, within the range of the experimental conditions, the slower the cooling the lower the blue value. A smaller influence is exerted by conditions of dissolving, standing at 90° lowering the value. Evidently, these conditions must be controlled if the blue value is to be used as a criterion.

From this and other experiments it appears that when amylose is unduly exposed to hot water a part may precipitate, this being irreversibly insoluble (retrogradation), whilst more may be changed but remain in solution. The solution now exhibits a lower blue value than before and can no longer be hydrolyzed to 100% of maltose by β amylase. The same is true of amylose which has been precipitated and washed by ethanol and ether. It is less soluble in water and the soluble portion has a lower blue value than before this treatment. This changed, soluble amylose is apparently related to the natural amylose much as denatured protein is to the corresponding native form. On the other hand, retrograded material would seem to correspond to protein which has been both denatured and coagulated. The blue value is not related to the mean chain

length of the molecules, but to the extent of denaturation. As amylose is purified it shows an increasing tendency to retrograde.

Recrystallization from hot aqueous solution by half saturation with butanol effects no fractionation of amylose so far as is indicated by blue values, provided that two conditions are fulfilled. The precipitated portion and that remaining in solution must each be separately recrystallized from hot butanol saturated water and redissolved under the same conditions, and there must be no denaturation. If there are any signs of retrogradation, such as marked opalescence, the unprecipitated fraction is found to have a lower blue value than the precipitate, e.g. 0.310 as against 0.340.

Purification and properties of amylopectin

Amylopectin was not further purified or its blue value lowered by reprecipitation by thymol, butanol, acetone, chloroform, or thymol cyclohexanol mixture in equal parts, by shaking to facilitate retrogradation of amylose or by filtration through cotton. The latter adsorbed added amylose, however. The blue value was not altered from that of the first amylopectin precipitate, although this value varied markedly according to the original method of precipitating the amylose. Thus, purer amylopectin was obtained by precipitation with thymol than with butanol, and still better with cyclohexanol followed by thymol.

Presumably, the amylose remaining entangled with the amylopectin consists of short molecular chains which the precipitant fails to pick out. Various precipitation procedures were tried. For example, ethanol was added to 1-2% of amylopectin in 0.05% NaCl until a cotton-like precipitate (I) was formed. This was separated and the mother liquor allowed to stand. In the course of the following days a further precipitate slowly formed

(ppt II). The results in Table 2 suggest that a little contaminated amylose was preferentially removed with the first precipitate, but neither preparation is considered to be entirely free from amylose. β -Amylase indicated no difference between the first and second precipitates, which hydrolyzed at the same rates and reached the same limits.

Amylopectin is in general hygroscopic. It adheres so strongly to glass or porcelain that in the course of drying the shrinking material picks particles of glass from the surface of the vessel. This is specially noticeable on cooling the dried amylopectin, the differences in thermal expansion of glass and amylopectin coming into operation.

SUMMARY

1 Amylose and amylopectin have been prepared from non autoclaved potato starch paste by successive additions on alternate days of cyclohexanol and thymol, which, on the whole, gave the best results, and by the methods of Schoch and of Haworth, Peat & Sagrott.

2 No great differences were noted among the products obtained by use of three precipitants.

3 There is no advantage to be gained by heating the starch paste under pressure. If this is omitted a fairly wide range of pH may be tolerated at the time of precipitation, but the amylose produced under acid conditions showed the greater tendency to retrograde in neutral solution.

4 Preparations of amylose with 17 times the blue value, as measured on the Spekker absorptiometer, of the corresponding amylopectin have been obtained.

5 The blue value of amylose is greatly influenced by the method of recrystallization from butanol-saturated water, and by the period of heating when redissolving.

Table 2 *Blue values of amylose and amylopectin*

Precipitant	pH at time of precipitation	Blue values			
		Amylose	Crude	Amylopectin	
				Ethanol precipitates	
				I	II
Butanol	4.1	0.285	—	0.028	0.025
	6.0	0.310	0.024	—	—
	9.4	0.320	—	0.027	0.025
Thymol	4.1	0.302	—	0.029	0.025
	6.0	0.332	—	—	—
	9.4	0.342	—	0.028	0.025
cycloHexanol followed by thymol	4.1	0.318	—	0.028	0.022
	6.0	0.336	0.021	—	—
	9.4	0.334	—	0.027	0.023

6 Exposure of amylose to hot water brings about changes even in the portion which does not retrograde. The blue value falls and β amylase action is incomplete.

7 Amylopectin was not so successfully purified, but fractional precipitation with ethanol freed it

slightly from amylose which had escaped the first precipitation.

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The Action of β -Amylase on Potato Amylose

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It has long been known that almost all natural starches contain a constituent which can be hydrolyzed to completion by β amylase. It has been given various names, that of amylose being the one used here. It is only in quite recent years that amylose has been prepared in a state of purity (Schoch, 1942, Haworth, Peat & Sagrott, 1946). β Amylase has usually been reported as promoting the hydrolysis of amylose to 90–100% of maltose, but many of these figures may be above the truth. Often, it has not been made clear whether the figure reported referred to maltose expressed as a percentage of the moisture free starch used or of the maltose theoretically obtainable from it. The absence of glucose from the products has been assumed, although many preparations of β amylase contain maltase. Lastly, it is rare that the β amylase has been demonstrated to be free from α amylase and from maltase.

In this work, the purest amylose and β amylases obtainable have been investigated with special attention to the kinetics of the reaction and a hypothesis is advanced to account for the facts.

MATERIALS AND METHODS

Substrates The various preparations of amylose are described elsewhere (Hopkins & Jelinek, 1948). Solutions not exceeding 0.9% concentration were prepared by heating the butanol amylose complex in water until the butanol was driven off.

Blue value (B.V.) The procedure of McCready & Hassid (1943) as modified by Hopkins & Jelinek (1948) was employed.

Red value (R.V.) This was measured in the same way as the B.V., except that filters 602 and 603 were used with the

Spekker absorptiometer. It was found of advantage, however, to increase the iodine concentration from 0.002 to 0.008%.

Enzyme preparations β Amylase was prepared (1) from well ripened barley as described by Hopkins, Murray & Lockwood (1946), (2) from soya beans by the method of Newton & Naylor (1939) and Newton, Hixon & Naylor (1943). Both specimens were tested and found to be free from maltase activity under the appropriate conditions of use. The limits of hydrolysis of starch pastes were 57% of the theoretical maltose ($R_m = 57\%$), and the preparations were considered to be free from α amylase.

Reaction mixtures These were as employed by Hopkins *et al* (1946), but contained varying concentrations of amylose as stated in the tables, and were buffered at pH 4.6 for barley and pH 5.8 for soya amylase. Progress of hydrolysis at 25° was determined as described by Hopkins *et al* (1946), except that the hypiodite titration was found not to be wholly satisfactory in the presence of certain antiseptics or if a trace of butanol was present. In such cases, the method of Cole (1933) was substituted, the ferricyanide being calibrated against maltose. Withdrawals from the reaction mixture were made into boiling water.

Calculation of the values of 'L' as if the reaction were monomolecular revealed that these decline progressively and markedly through the reaction. As is shown later, there was no theoretical reason for expecting monomolecularity.

For the determination of the affinity constant, a series of flasks was set up containing varying concentrations of the amylose (about four flasks for each concentration). All flasks contained identical concentrations of enzyme and buffer. After suitable intervals of time (estimated from a pilot experiment) pH 10 buffer was added to the respective flasks to check the reaction and the maltose determined by titration. The velocity of reaction v (mg maltose/100 ml/min) was obtained for each concentration of amylose substrate from the initial slope of the progress curves.

RESULTS

The limit of action was usually 99% R_m , the full 100% being obtained in some cases. An example is given in Table 1. The final reaction mixture from this experiment was concentrated after distilling off traces of antiseptics. The reducing power in a sample was determined by hypiodite titration. After making allowance for weight and reducing power of the enzyme, the results indicated pure maltose $[\alpha]_D^{15}$ was found to be +136°

Table 1 Action of β amylase (soya) on potato amylose (B v 0 332)

(Concentration of amylose in reaction mixture 0.41%)

Reaction time (hr)	% of theoretically obtainable maltose (R_m)	Reaction time (hr)	% of theoretically obtainable maltose (R_m)
0.5	49.3	3.0	85.4
1.00	62.7	3.5	88.4
1.33	68.5	4.0	89.3
1.5	71.0	5.0	92.8
1.75	74.1	20	97.6
2.0	76.4	90	99.1
2.5	82.2	102	100.0

Amylose prepared from autoclaved starch paste (120° for 2 hr at pH 7, Table 2, no. 1) underwent hydrolysis about 1.5 times as fast as that similarly obtained from paste prepared at 90° (no. 2), but

each reached the same limit. It may be remarked that the amylopectins prepared at the same time underwent hydrolysis at substantially the same rate as one another.

The reaction of the starch paste at the time of crystallization of amylose influenced the rate at which the amylose subsequently became hydrolyzed at pH 5.8. Of the amyloses in Table 2, one series, nos. 3, 4 and 5, had been precipitated respectively at pH 4.1, 6.0 and 9.4 by butanol, whilst nos. 6 and 7 constituted a pair precipitated at pH 4.1 and 9.4 respectively by cyclohexanol followed by thymol. The choice of precipitant proved to be relatively unimportant, but the amyloses precipitated at acid reaction underwent hydrolysis the most rapidly. Associated with their liability to retrograde, discussed elsewhere (Hopkins & Jelneek, 1948), they failed to reach as advanced a limit of hydrolysis as the other samples. No. 3 partly came out of solution in hot water on two occasions before enzyme action was commenced. The substrate remaining in solution was hydrolyzed to an even lower limit (no. 8) than the whole sample (no. 3). During heating, some had precipitated, but more had been changed ('denatured') into a form less susceptible to β amylase. The purer, i.e. the more 'native' the amylose, the more completely it is hydrolyzed and the higher its blue value (Fig. 1).

The results in Tables 1-3, e.g. nos. 1 and 2 of

Table 2 Action of β amylase (soya) on potato amyloses

(Only reaction mixtures of a given series as mentioned in the text received identical quantities of the enzyme)

(Results as percentage of theoretically obtainable maltose)

No. of amylose sample (see text)	1	2	3	4	5	6	7	8	9
Feature of its preparation	Paste heated at 120°	Paste heated at 90°	Pre cipitated at pH 4.1	Pre cipitated at pH 6	Pre cipitated at pH 9.4	Pre cipitated at pH 4.1	Pre cipitated at pH 9.4	No. 3 partly retro graded	—
Blue value	0.322	0.332	0.285	0.310	0.342	0.308	0.326	0.252	0.327
% Amylose in reaction mixture	0.61	0.45	0.58	0.59	0.58	0.25	0.26	0.42	0.40
Time									
10 min	25.0	19.5	19.9	14.8	12.8	20.1	13.3	26.0	—
20 min	44.4	29.3	—	24.2	—	37.0	22.4	—	—
30 min	—	—	—	—	—	—	—	47.4	—
40 min	52.8	37.9	53.2	37.2	32.5	54.0	37.2	—	—
60 min	61.7	46.7	—	46.4	40.1	63.9	46.0	—	—
90 min	69.4	55.6	—	55.1	47.6	—	—	—	—
120 min	73.8	—	—	60.3	54.2	81.6	62.3	—	—
150 min	—	64.7	—	—	—	—	—	—	13.4
180 min	79.6	66.7	—	69.5	62.6	86.0	69.3	—	—
210 min	81.3	72.0	—	—	—	—	—	—	—
240 min	82.4	74.0	—	—	—	87.3	75.1	—	—
300 min	83.1	77.4	—	76.0	72.0	88.3	82.2	78.0	25.7
360 min	84.4	80.4	—	—	—	—	—	—	29.6
435 min	—	—	—	—	—	—	—	—	33.8
405 min	—	—	—	—	—	—	—	—	34.9
11 hr	—	—	—	—	—	—	—	—	44.2
24 hr	—	—	91.5	91.5	95.9	92.5	95.0	82.5	62.2
Limit	97.4	98.0	94.0	—	98.0	94.8	99.0	85.5	—

Table 3, and the curves *A* and *B* of Fig 1 show that the rate of hydrolysis is never constant but declines steadily. Except in the case of the partly 'denatured' amylose samples (e.g. nos 3 and 6 of Table 2) there is no sudden break in the curve if percentage maltose is plotted against time. Thus

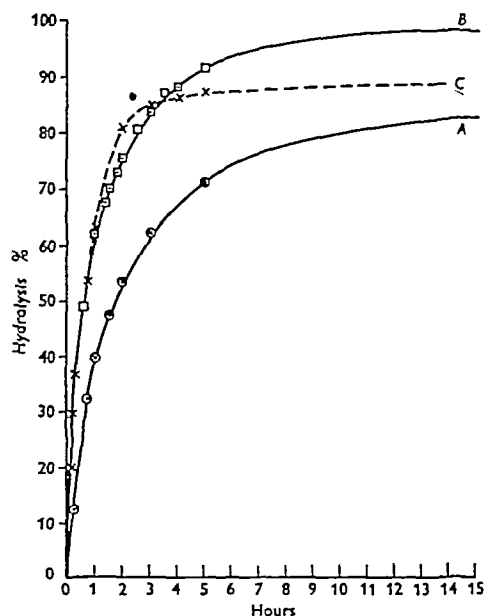


Fig 1 Progress of hydrolysis of amylose by β amylase (soya). The curves beyond 5 hr were interpolated, making use of later readings which are not within the range of the graph. Curve *A* From Table 2, no 5 Limiting % 98. Curve *B* From Table 1 Limiting % 100. Curve *C* From Table 2, no 6 (partly denatured amylose) Limiting % 95.

means that there is no evidence from our results of any branched or laminated chains in amylose, as has been suggested by other workers who observed

such breaks. These do occur, however, with partly 'denatured' amylose samples, and the hydrolysis does not reach the full 100% (Fig 1, curve *C*).

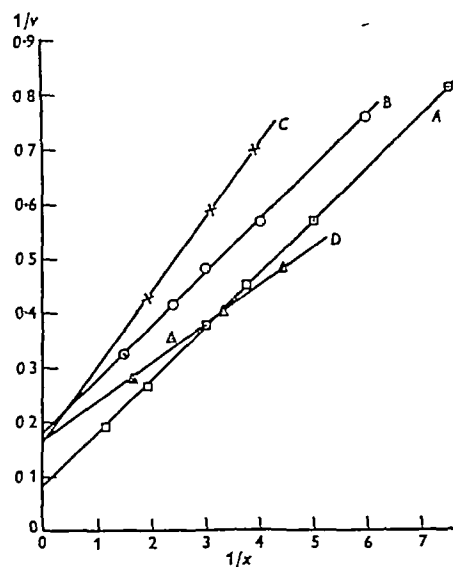


Fig 2 Initial velocity substrate concentration relationship plotted according to the procedure of Lineweaver & Burk (1934) for the system β amylase (soya) amylose. For amylose preparations *A*–*D* inclusive, see text.

	V (max velocity) (mg maltose produced/100 ml/min)	K_m
<i>A</i>	12.0	1.17
<i>B</i>	5.45	0.53
<i>C</i>	6.25	0.86
<i>D</i>	6.05	0.45

Returning to the steady decline in velocity, this is more pronounced than if the reaction were monomolecular. At the same time the β amylase amylose

Table 3 Action of β -amylase (barley) on potato amylose

(Results as percentage of theoretically obtainable maltose)

No. of sample (see text) Feature of its preparation	1 Precipitated by thymol (not autoclaved)	2 Precipitated by butanol (not autoclaved)	3 Mixed samples
Blue value	0.345	0.355	0.336
% Amylose in reaction mixture	0.72	0.72	0.63
Time			
15 min	7.5	—	22.2
30 min	12.2	11.9	32.0
45 min	16.4	—	—
60 min	20.1	20.0	45.6
90 min	26.3	—	53.2
120 min	31.7	34.0	58.9
180 min	—	44.4	66.8
240 min	52.4	53.8	72.2
300 min	—	61.4	75.8
360 min	—	68.5	79.2
420 min	—	74.2	—
480 min	—	77.7	—
24 hr	97.1	—	94.8
Limit	99.5	100.0	100.0

system complies with the Michaelis & Menten formulations in certain respects, since on plotting reciprocals of velocity ($1/v$) against those of concentration ($1/x$) straight lines were obtained (Fig 2). The values of K_m (the affinity constant) and V (the maximum velocity attainable) were deduced by the usual procedure (Lineweaver & Burk, 1934). It is interesting to note that the affinity constants varied with specimens of amylose according to the conditions of precipitation. The 'amylose A' (Fig 2) was precipitated from potato paste prepared at 100° by the addition of cyclohexanol (0.15%) and thymol (0.15%) simultaneously at 25° following the sug-

Michaelis formula. This is associated probably with 'denaturation' of the amylose under such conditions.

When amylose is undergoing hydrolysis with β -amylase the molecular chains are being progressively shortened. By withdrawing a portion of the reaction mixture, stopping the hydrolysis in it and recovering the residual polysaccharide by the butanol precipitation method, specimens were obtained of amylose in such stages of degradation. These in turn, when set up in identical reaction mixtures, were found to be hydrolyzed at widely different rates, the less degraded the amylose, the faster it underwent hydrolysis (Table 4). The partly

Table 4 Action of β amylase on amylose, previously partly degraded

(Results as percentage of theoretically obtainable maltose)

No. of sample (see text)	Original	E	F	G
Blue value	0.327	0.335	0.327	0.339
% Substrate in reaction mixture	0.324	0.322	0.330	0.316
Time				
12 min.	24.0	—	20.6	15.6
22 min.	—	37.7	30.0	21.8
44 min.	58.5	51.6	44.4	31.7
60 min.	—	57.7	47.6	32.6
90 min.	—	62.0	—	—
150 min.	—	73.5	60.9	44.9
210 min.	—	79.2	69.6	51.0
300 min.	—	85.9	75.5	55.2
20 hr.	91.8	95.5	89.9	85.3

gestion of Haworth *et al* (1946). Amylose separated in 3–5 days and was removed ('amylose A'). The residual paste was then heated to 100° and cooled slowly, more amylose separating ('amylose B'). Each amylose was purified as described by Hopkins & Jelínek (1948). The first fraction to precipitate (A) gave the higher value of K_m , but as K_m is expressed as a percentage and not as molarity (the mean molecular weight being unknown) this does not necessarily mean that this amylose possessed less affinity for β amylase than the other. Evidence to be cited later indicates that the larger the amylose molecules the faster do they hydrolyze. This agrees with the high value of V for the first precipitate, the assumption being that it was the longer chain molecules which aggregated and came out of the paste the more readily.

Specimens C and D (Fig 2) refer to two amylose 'fractions' prepared from one purified sample by partial saturation of its hot aqueous solution with butanol and slow cooling. Half of the amylose (C) separated from solution and was centrifuged off. The rest was recovered by full saturation (D). Again, the more precipitable fraction gave the higher value of K_m and of V . Evidently the numerical value of K_m for amylose and β amylase varies widely according to conditions of preparation. It may bear a relationship to mean chain length. There are indications that in relatively concentrated solutions of amylose, v does not increase as predicted from the

hydrolyzed specimens were obtained by withdrawal from reaction mixture no. 9 of Table 2, E at 13.9%, F at 44.2% and G at 69% (after 46 hr). It seems clear that the further amylose is degraded by β -amylase, the more difficult it becomes for the amylose to hydrolyze it, assuming that no change in molecular character has occurred during the recovery of the partly hydrolyzed fractions.

For the experiments reported in Table 5 enzyme action was checked by withdrawing samples and running into alkali instead of boiling water, since boiling may change the blue value of amylose. The percentage hydrolysis was determined by hypodermic, and, after calculation of the concentration of residual polysaccharide, the liquid was diluted and used for the measurement of blue and red values. It was ascertained that these values were not influenced by the presence of maltose or the enzyme preparation. Samples H and K were prepared from amylose in the same way as B and A respectively of Fig 2. Sample H was a normal sample, whilst K had been very slightly 'denatured' as shown by its behaviour.

Filters 602 (4300–4700 Å transmission) and 603 (4700–5100 Å) may be regarded as transmitting light which will be absorbed by red liquids. Readings in the Spekker absorptiometer using these filters are therefore 'red values' and will be relatively high in the presence of amylopectin or of dextrans of chain length about 7–12 glucose units. On the other

hand, with filter 607 (5900-6300 Å) scarcely any absorption by red solutions is measured, and with filter 608 none at all. With the latter filters, the absorption by amylose of greater mean chain length than 12 is measured. Only the values with filters 603 and 608 are recorded, but in several experiments a close parallel was observed between the 602 and 603 readings and also between the 607 and 608 readings.

expected since the formation of the reaction product, maltose, is not accompanied by a corresponding diminution of the molar concentration of the substrate. The percentage concentration of the latter diminishes, yet the molar concentration remains unaltered until one or more chains in the system become too short for further hydrolysis, either in the form of maltose or a trisaccharide. If all amylose molecules were attacked with equal

Table 5 *Changes in blue and red values of residual polysaccharide during hydrolysis*

(Hydrolysis in percentage of theoretically obtainable maltose)

Sample % Amylose in reaction mixture	H (normal sample) 0.64				K (slightly 'denatured') 0.62			
	Hydrolysis	Blue value (filter 608)	Red value (filter 603)	Ratio 608/603	Hydrolysis	Blue value (filter 608)	Red value (filter 603)	Ratio 608/603
Time (min)								
0	0	0.346	0.131	2.64	0	0.327	0.127	2.56
40	56.0	0.327	0.130	2.52	53.4	0.282	0.117	2.41
70	66.5	0.310	0.120	2.40	61.1	0.258	0.107	2.41
100	74.3	—	—	—	67.6	0.247	0.102	2.42
130	78.6	0.280	0.120	2.33	72.2	0.230	0.100	2.30
160	82.0	0.255	0.114	2.24	75.2	0.199	0.090	2.21
240	88.6	0.220	0.109	2.02	81.9	0.164	0.077	2.13
300	92.2	0.179	0.100	1.79	86.0	—	0.066	—
360	93.8	0.128	0.084	1.53	88.0	0.084	0.048	1.75
420	95.0	0.078	0.069	1.13	89.8	0.040	0.039	1.03
500	96.9	0.059	0.076	0.77	90.7	0.021	0.032	0.66

DISCUSSION

In explanation of the various observations reported above the following hypothesis is advanced. Amylose consists of a mixture of chain molecules of various lengths, i.e. of a homologous series, the majority, according to the law of distribution, being of intermediate length. All but the relatively very short ones possess approximately the same blue value. Fractional precipitation, both from starch paste and from purified amylose, brings out the longer chains first, the shorter needing more drastic conditions to pick them out from the amylopectin, or to aggregate and crystallize them from aqueous solution. Heating changes the coiled pattern of the native macromolecules and renders them less blue in presence of iodine, less soluble in water and less completely hydrolyzable by β amylase. With prolonged heating irreversible retrogradation occurs.

The longer chains are attacked preferentially and the more rapidly by β amylase. The action of β amylase is in one respect different from most enzymic reactions in which a single substance is decomposed. Successive maltose molecules are formed by the removal of two glucose groups at a time from the non-reducing end of the chain, a molecule of polysaccharide and a molecule of maltose being formed in each individual reaction. The more usual kinetics of an enzyme reaction cannot be

readiness, irrespective of chain length, a zero order reaction would be expected, whatever the concentrations of substrate and enzyme employed. This would persist until the shortest chains were exhausted and, unless the substrate was suitably dilute, until inhibition by reaction products or inactivation of the enzyme interfered. A zero order reaction, however, is never observed, not even with the highest concentrations of substrate employed.

On the other hand, if we assume that the longer the chain the faster it is hydrolyzed, we should expect a steadily declining rate of reaction, as is invariably observed. It could not be of zero order over any appreciable percentage of hydrolysis. But there is no reason to suppose that it would be monomolecular. With low concentrations of substrate and fast reactions the value of k should actually fall, as was usually observed. A consideration of the results in Table 4 will make this clear. Amylose *G* must have possessed a mean chain length of about one third of that of *E*, since it had previously been hydrolyzed by β amylase to 69%, compared with 13% for *E*. As each was now freshly hydrolyzed at the same percentage concentration, the molarity of *G* in the reaction mixture must have been about three times that of *E*. At the concentration employed, 0.32%, the latter would largely control the rate of maltose formation, as may be deduced from the affinity constants. We should therefore expect *G* to be hydrolyzed at something like three times the

rate of E , but it was hydrolyzed at about half the rate. With decreasing chain length the rate of β amylase action falls off more than proportionately.

It appears that β amylase combines with the substrate, and as regards the initial velocity of the reaction it conforms with the Michaelis & Menten theory. Owing, however, to the non homogeneous nature of the substrate, the subsequent time progress relationship is not that usually associated with hydrolytic enzyme actions which obey the Michaelis & Menten relationship. It is a case of several substrates competing for the same enzyme.

It is assumed that the affinity constant, K_m , varies according to the mean chain length of the amylose, and when expressed as molarity decreases with increase of chain length. But the effect of increase of chain length on the value of K_m , expressed as percentage, would be to increase it simultaneously in proportion to molecular weight.

To distinguish between samples of amylose of different mean chain length by β amylase action, a low concentration of substrate is desirable so that the latter controls the reaction velocity. It was for this reason that such conditions were imposed when amyloses precipitated under different conditions, or previously hydrolyzed to various stages, were under comparison. In the latter case, samples F and G (Table 4), being partly hydrolyzed, must have had relatively low mean chain lengths, and therefore relatively high molar concentrations compared with E . Nevertheless, they were hydrolyzed much more slowly. This experiment, moreover, eliminated the complication of inhibition of enzyme action by maltose.

We may therefore conclude that β amylase attacks more readily the longer chain molecules and, provided no denaturation has taken place, hydrolysis proceeds at a steadily declining rate with no sudden change, continuing until 100% maltose has been formed.

When amylose is hydrolyzed by α amylase or acid, the iodine coloration passes from the blue through purple and red to colourless. We have results indicating that the colourless stage corresponds to dextrins composed of about 6 glucose units, and the red stage to 10 units or so. This is supported by certain observations on synthetic products reported by Cori, Swanson & Cori (1945). When, however, β amylase is used some blue colour remains until the hydrolysis has advanced well beyond 90%.

The relationship between the blue value and mean chain length of amylose is indicated by the results in Table 5. Whilst blue value is zero for short chains, it becomes measurable at about chain length 12, and increases rapidly with chain length. Later it approaches an asymptotic value (cf. specific optical rotation). Conversely, as the chain length is reduced by β amylolytic action, blue value declines but little during the first 70% hydrolysis, but later falls more rapidly, disappearing at about 95%. The red value is slightly

later in falling, otherwise its behaviour is similar. Most, if not all, of the red value of the original amylose is due to the same colouring matter as the blue value and is numerically proportional to it. The ratio 608/603 reading remains approximately constant for 75% of the hydrolysis, which would scarcely be expected if there were present in the original amylose a separate fraction such as short chain material, giving a red colour with iodine. However, at the point where material giving blue with iodine disappears through hydrolysis, the blue value would rapidly decline, whilst the corresponding decrease in red value would be delayed. Indeed, the latter might, in certain circumstances increase numerically for a time, depending on whether short-chain material giving a red iodine colour were formed fast enough to more than counterbalance the loss of red colour due to destruction of iodine blueing polysaccharide. In at least one experiment a small rise in red value at about 80% was observed. However, owing to the possibility of appreciable error creeping in when computing the concentration of residual polysaccharide at late stages of the hydrolysis neither the blue nor the red values in these late stages should be taken as accurate. Their ratios are significant, since the blue and red readings were taken on the same diluted material, and the above source of error is cancelled out. The ratio of 608/603 reading falls markedly after 80% hydrolysis, indicating the disappearance of amylose of more than 12 units chain length and generally supporting the conclusions drawn above. Apparently 80-90% or more of amylose is hydrolyzed before the mean chain length is as low as 10-12. Some specimens of amylose gave somewhat different curves from those drawn from the data in Table 5 although of the same type. Sometimes there was no appreciable fall in blue or red value till 80% hydrolysis. Sample K in Table 5 was a slightly 'denatured' amylose. The residue, when just over 90% hydrolysis was attained, possessed no blue or red colouring power, and was able to resist hydrolysis.

SUMMARY

1. Pure potato amylose is hydrolyzed by β amylase at a steadily declining rate, without sudden velocity changes, until 100% of maltose has been formed.

2. If any 'denaturation' of the amylose has occurred, due to undue exposure to water, there is a break in the progress curve and the hydrolysis is incomplete.

3. Amylose crystallized from potato starch paste at pH 4, or from autoclaved paste at ordinary reaction, hydrolyzes faster than preparations crystallized from non autoclaved pastes at pH 6 or 9.

4. The reaction obeys the formulation of Michaelis & Menten, but the value of K_m (expressed as percentage) varies widely with the preparations.

5. The shorter the molecular chains the more slowly they are hydrolyzed.

6. Evidence from iodine colorations suggests that amylose prepared by the crystallization methods contains no short chain molecules (chain length < 12), and that none is formed until hydrolysis is far advanced.

7 The blue value of amylose increases with chain length, approaching an asymptotic value. Conversely, it decreases on hydrolysis.

8 A hypothesis is suggested to account for the observed kinetics.

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The Distribution and Interrelationships of the Alkaloids in the Bark of *Cinchona Ledgeriana*

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The literature on the interrelationships between the principal alkaloids and their distribution throughout the bark of trees of the cinchona species is scattered and rather sparse. The references available indicate that in general the alkaloid content is greater in the root bark and at the base of the tree than in the upper parts. Hodge (1946) found, however, that the reverse condition obtains in some wild Peruvian cinchonas. Sando (1944), who studied alkaloidal distribution in *Cinchona succirubra*, found a positive correlation between alkaloid content and tree girth.

The four main crystallizable alkaloids in the bark are the two groups of stereo-isomerides quinine and quinidine, and cinchonidine and cinchonine. Quinine and quinidine contain a methoxyl group in the 6-position of the quinoline nucleus, otherwise the molecular structures are the same. Such similarity in structure suggests a common mechanism of formation. The proportions in which the alkaloids are laid down in the bark, the wide differences in the proportions of the alkaloids according to bark species, and the smaller differences found among barks of the same species, and in the bark from various parts of a single tree are probably due to the operation of factors affecting the rates of conversion of one alkaloid to another.

The present investigations were undertaken to determine the distribution of the alkaloids (total,

individual and amorphous) throughout the whole bark, and to relate this distribution with the form of the tree, the metric characteristics of the bark and the proportions of the various alkaloids. Some interesting relationships have been established.

EXPERIMENTAL

Materials

The trees selected were of the Ledger species, approximately 7 years old and grown on the same plantation. Bark from *C. Ledgeriana* is the richest in quinine of all cinchona barks, and contains usually 8-12% of alkaloids, of which 3-8% is quinine.

Tree 2137 was felled at ground level and the trunk sawn into 25 cm sections over a length of 4 m from the base. The main stem was divided into two, 2 m from the base. The barks from sections equidistant from the base along each of these stems were combined. The bark from each section was air dried, weighed and ground to pass a 50 mesh sieve. The root bark was collected as one sample. The sections were numbered consecutively from the base upwards.

Tree 1970. The preliminary investigations on tree 2137 showed that the alkaloid distribution is to some extent a function of the form of the tree. A detailed drawing was therefore made of tree 1970 showing the location of secondary leaders (erect growing stems as opposed to more or less horizontal branches) as well as of living, dead and, as far as possible, former branches. The tree was of irregular girth with maximum trunk thickness about 130 cm above ground, where there was also a marked bending of the trunk from the vertical for a distance of about 20 cm (Fig 1).

The main stem and the secondary leaders were cut into 10 cm sections. The lateral branches were divided into four groups (primaries, secondaries, tertiaries and quaternaries) and the leaves into seven groups ranging from very young to mature. In the lower parts of the tree one section was selected at random from each 50 cm length, except where the formation of the tree (as revealed by the preliminary

Analytical methods

The samples were analyzed for total alkaloids (TA), quinine (Q), cinchonidine (Cd), cinchonine (C), and amorphous alkaloids (Am), and the results expressed as a percentage of the moisture free bark. Quinidine was present in traces only and was not quantitatively determined.

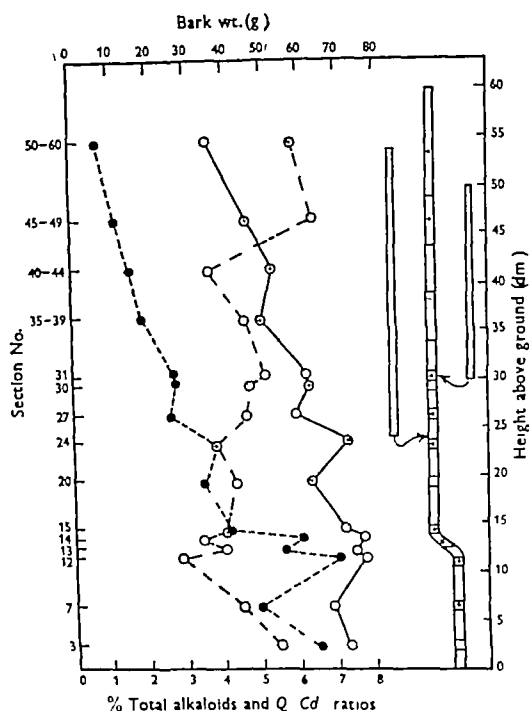


Fig 1 Tree 1970 Diagram showing the form of the tree with the locations of the secondary leaders and the sections analyzed. The corresponding analytical data/10 cm section are plotted on the left. ● — ● Bark weight, ○ — ○ percentage total alkaloids, ○ — — — ○ Q Cd ratio. Average values of bark weight/10 cm section are given for each of the four groups of composite samples above section 35.

work on tree 2137) was of special interest, e.g. where the main stem bent, or where big lateral branches arose. At such positions two or three contiguous sections were taken. Where the amount of bark from a 10 cm section was insufficient for analysis the bark from a sequence of five sections was bulked, i.e. a 50 cm length of stem. For the lateral branch bark and leaves a composite sample was taken from each of the groups into which they had been divided. Prior to preparing the samples for analysis, the thickness, area, volume and weight (air-dry) of the bark from each section were measured, also the girth of each section and the corresponding areas of the trunk.

Tree 1512 was selected, in contrast to tree 1970, for its straggled form and apparently normal growth (Fig 2). It had produced one secondary leader only. A detailed drawing was made of the tree and the same sampling technique employed as that for tree 1970.

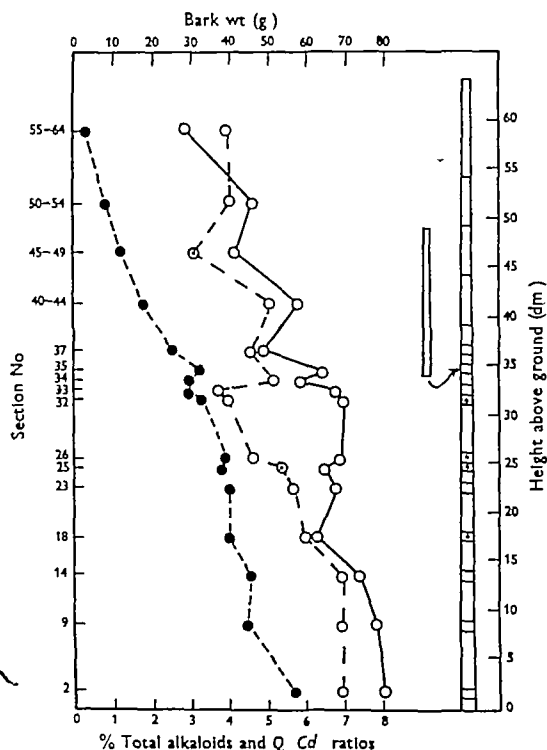


Fig 2 Tree 1512 Diagram showing the form of the tree with the locations of the secondary leader and the sections analyzed. The corresponding analytical data/10 cm section are on the left. ● — ● Bark weight, ○ — ○ percentage total alkaloids, ○ — — — ○ Q Cd ratio.

Total alkaloids Air-dry bark (20 g) was mixed with CaO (12 g), water (15 ml) and 5N NaOH (2 ml). After standing overnight the mixture was extracted with benzene in a Soxhlet apparatus. The alkaloids were recovered from the benzene extract with 5% (w/v) H_2SO_4 , the latter was then made alkaline and the liberated alkaloids extracted with chloroform. The bulk of the chloroform was distilled, and the residual extract washed with chloroform into a small tared beaker. After the addition of a small quantity of ethanol the solution was evaporated and dried to constant weight at 100° .

The individual alkaloids in the total alkaloids were determined by the *British Pharmacopoeia* (1932) method for totaquina.

RESULTS

The analytical data not shown in the figures are given in Table 1.

Table 1 *Analytical data for trees 2137, 1970, 1512*

Tree no	Bark section no	Alkaloids (% dry bark)			
		Quinine	Cinchonidine	Cinchonine	Amorphous alkaloids
2137	1	6 75	1 20	0 70	1 11
	2	7 44	1 19	0 65	1 09
	3	6 99	1 11	0 70	1 00
	4	7 48	1 41	0 96	1 08
	5	7 32	1 30	0 80	0 82
	6	6 67	1 06	0 74	1 38
	7	6 82	1 22	0 90	1 17
	8	5 87	1 93	1 47	1 25
	9	6 51	1 47	0 90	0 80
	10	6 21	1 35	0 93	0 96
	11	5 31	1 38	0 78	0 90
	12	5 09	0 85	0 62	1 01
	13	6 21	1 42	0 89	1 03
	14	6 01	1 19	0 83	0 85
	15	5 06	0 62	0 71	1 04
	16	5 14	0 97	0 67	0 99
1970	Root bark	5 68	1 60	1 81	1 22
	3	4 86	0 80	Trace	1 16
	7	4 58	0 02	Trace	0 97
	12	4 64	1 47	0 22	0 97
	13	4 77	1 09	0 31	0 88
	14	4 84	1 26	0 29	0 86
	15	4 23	0 94	0 11	1 30
	20	3 18	0 67	0 09	1 78
	24	4 66	1 11	0 19	0 82
	27	2 91	0 58	0 09	1 75
	30	3 07	0 59	0 11	1 92
	31	2 94	0 53	0 13	1 86
	35-39	2 86	0 56	0 10	1 00
	40-44	2 41	0 59	0 08	1 87
	45-49	1 82	0 26	0 02	1 39
	50-60	1 79	0 28	0 03	0 96
1512	2	5 38	0 71	Trace	0 91
	9	4 79	0 63	Trace	1 26
	14	3 77	0 50	Trace	1 19
	18	3 07	0 46	Trace	1 43
	23	2 61	0 42	Trace	2 04
	25	1 62	0 27	Trace	2 31
	26	3 50	0 69	Trace	1 90
	32	3 07	0 70	Trace	1 54
	33	2 63	0 03	Trace	2 08
	34	2 56	0 45	Trace	1 38
	35	—	—	—	—
	37	2 69	0 53	Trace	1 04
	40-44	2 40	0 43	Trace	1 43
	45-49	1 34	0 39	Trace	1 30
	50-54	1 21	0 27	Trace	1 80
	55-64	0 64	0 15	Trace	0 97

Tree 2137 The distribution of the total alkaloids in the bark is shown in Fig 3. There was an overall but irregular decrease of total alkaloid content from the base of the tree upwards. The maximum alkaloid content was found 2 m from the base (section 8) where the tree forked, with secondary maxima (sections of high alkaloid content relative to adjacent sections) at sections 4 and 13. Unfortunately, nothing had been recorded of the form of this tree at these levels.

Fig 3 shows also a positive correlation between percentage total alkaloids and bark weight/section.

The regression equations (Table 2) show that the percentage of total alkaloids, as well as the percentages of quinine and cinchonidine, were positively and significantly related to bark weight. The regression of cinchonine on bark weight was possibly significant, and that of amorphous alkaloids not significant.

These equations are more fully discussed later, but it should be noted here that increases in bark weight were associated with significant increases in the percentages of each of the crystallizable alkaloids. The equations give, however, only a general picture of

the association between increasing bark weight and increasing alkaloid content. When the molecular ratios quinine to cinchonidine (Q/Cd) and quinine to cinchonine (Q/C) were calculated for each section, an inverse relationship was found between these ratios and bark weight/section (Fig 3)

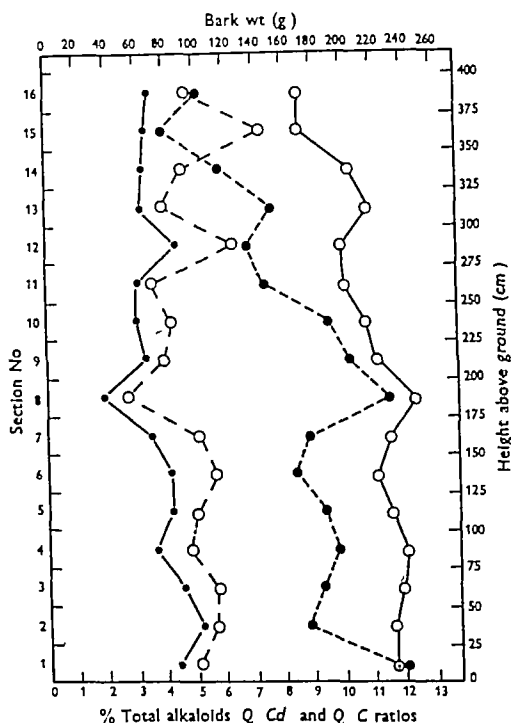


Fig 3 Tree 2137 The relationships between bark weight, percentage total alkaloids, the quinine to cinchonidine (Q/Cd) and quinine to cinchonine (Q/C) ratio/25 cm section of bark. The Q/C ratios shown are half the true values. ● —● Bark weight, ○ — — —○ percentage total alkaloids, ○ — — —○ Q/Cd ratio, ● — — —● Q/C ratio

There was a marked similarity in the alkaloid contents of the root bark, and the bark where the tree forked. In these samples the percentages of cinchonidine and cinchonine were much higher, and the Q/Cd , and Q/C ratios much lower than in samples from any other part of the tree. The quinine contents at the two locations were also similar. Quinine content was at a maximum in the bark from the lower stem roughly at about 1 m above the base. The amorphous alkaloid content showed little variation throughout the length of the tree.

Tree 1970 The form of this tree is shown in Fig 1 with the distribution of the total alkaloids, the weight of bark from the various sections and the Q/Cd ratios. As with tree 2137 the decrease of alkaloid content from the base of the tree up was

irregular, and wide fluctuations were associated with certain parts of the tree, in this case notably where the trunk bent and where the lower secondary leader arose. Zones of low alkaloid content were found at those parts of the trunk where there was no indication that branches were ever formed.

The regression equations (Table 2) show that the percentage of total alkaloids was positively and significantly related to the weight, thickness and volume of the bark. The percentages of quinine and cinchonidine, and possibly that of cinchonine, were likewise related to bark weight and thickness, while a negative regression, possibly significant, was found for the amorphous alkaloids. When the regressions of the percentage individual alkaloids/section on percentage total alkaloids/section were calculated, significant positive regressions were found for the three crystallizable alkaloids, and a not significant negative regression for the amorphous alkaloids (Table 2).

A further analysis of the data showed significant negative regressions for amorphous alkaloids on quinine and on cinchonidine, and very significant positive regressions for cinchonidine on quinine and cinchonine on cinchonidine (Table 3). When the individual alkaloids were expressed as percentage of the total alkaloids, the two negative regressions of amorphous alkaloids on quinine and on cinchonidine were very significant (Table 3).

With this tree, as with tree 2137, the Q/Cd and Q/C ratios were inversely related to bark weight or thickness (Fig 1). A well marked inverse relationship between bark weight and the Cd/C ratios was also noted, an observation not made with tree 2137, throughout the length of which the Cd/C ratios in the bark were fairly constant. The highest percentages of cinchonidine and cinchonine, and the lowest Q/Cd and Q/C ratios, were found where the tree bent and where the lower secondary leader arose. At the same places the quinine to amorphous alkaloid ratios were highest. The greatest percentages of quinine were also found here and at the base of the tree.

In the lateral branches there was a rapid fall in the alkaloid content of the bark, from the primaries to the tertiaries from 2.19 to 0.46% for total alkaloids, from 0.68 to 0.11% for quinine, and from 0.29 to 0.09% for amorphous alkaloids. Large scale extractions of the leaves, separated into groups ranging from young to mature, showed total alkaloid contents varying between 0.055 and 0.223%. No correlation was found between age of the leaf and total alkaloid content, and no crystallizable alkaloids could be isolated.

Tree 1512 The shape of this tree, the distribution of the total alkaloids, the related bark weights/section, and the corresponding Q/Cd ratios are shown in Fig 2.

Taken in conjunction, Figs 1 and 2 show how total alkaloid distribution is related to the form of the tree. In the lower half of tree 1512 the decrease in alkaloid content from the base upwards was, with relatively minor fluctuations, a gradual one. The first evidence of any living branches was at section 32, and between this and section 34, where there were no branches, the alkaloid content fell rather rapidly, but rose again at section 35 where the only secondary leader arose. Thereafter the alkaloid content decreased, but with two secondary maxima for the bulk samples taken from sections 40-44 and 50-54. These two sections had, on an average, a larger number of lateral branches than sections 45-49.

The regressions of percentage total alkaloids/section on bark weight, thickness and volume/section (Table 2) were of the same high order of significance as those for tree 1970. The interrelationships between bark weight, tree form and total alkaloid distribution appear therefore to be fairly well established.

Increases in bark weight were also associated with significant increases in the percentages of quinine and cinchonidine, but the increase in amorphous alkaloids was not significant (Table 2). Increases in percentage quinine were accompanied by significant increases in cinchonine, but no significant relationship was found between these two alkaloids and the amorphous alkaloids (Table 3). When, however, the alkaloids were expressed as a percentage of the total alkaloids a highly significant negative regression was found for amorphous alkaloids on quinine, and a possibly significant negative regression for amorphous alkaloids on cinchonidine (Table 3).

In contrast to trees 1970 and 2137 the bark from tree 1512 contained insufficient cinchonine for a quantitative determination. Moreover, the Q/Cd ratios of the samples taken from the lower half of the tree decreased from the base of the tree upwards. At sections 26, 32 and 33, where the Q/Cd ratios were relatively low, there were, respectively, two dead branches, two living branches and a dead and

Table 2 *Regressions of the total and individual alkaloids on bark weight, thickness and volume, and of the individual alkaloids on total alkaloids*

(Y_{TA} , Y_Q , etc. are the calculated percentages of total alkaloids, quinine, etc. per section for any value of X (bark wt., g), X_1 (bark thickness, mm), X_2 (bark volume, ml) or X_3 (percentage total alkaloids), per section, lying within the range of values found for the sections analyzed. P shows the level of significance of the regression. N.S. not significant.)

	Tree no 2137 (25 cm section)	P	Tree no 1970 (10 cm section)	P	Tree no 1512 (10 cm section)	P
Y_{TA}	$7.14 + 0.0223 X$	0.01	$4.220 + 0.0565 X$	0.01	$3.47 + 0.084 X$	0.01
Y_Q	$4.45 + 0.0114 X$	0.01	$1.645 + 0.0523 X$	0.01	$0.371 + 0.077 X$	0.01
Y_{Cd}	$0.42 + 0.0048 X$	0.01	$0.210 + 0.0154 X$	0.01	$0.256 + 0.007 X$	0.01
Y_C	$0.45 + 0.0022 X$	0.10	$0.031 + 0.0024 X$	0.10	—	—
Y_{Am}	$0.90 + 0.0008 X$	N.S.	$1.654 - 0.0096 X$	0.10	$1.453 + 0.002 X$	N.S.
Y_{TA}	—	—	$0.88 + 1.0885 X_1$	0.01	$0.40 + 1.320 X_1$	0.01
Y_{TA}	—	—	$4.25 + 0.0162 X_2$	0.01	$3.16 + 0.036 X_2$	0.01
Y_Q	—	—	$-1.870 + 0.864 X_3$	0.01	—	—
Y_{Cd}	—	—	$-0.814 + 0.253 X_3$	0.01	—	—
Y_C	—	—	$-0.170 + 0.046 X_3$	0.05	—	—
Y_{Am}	—	—	$1.914 - 0.098 X_3$	N.S.	—	—

Table 3 *Regressions of the individual alkaloids per section A for the alkaloids expressed as percentage of the bark, B for the alkaloids expressed as percentage of the total alkaloids*

(Y_{Am} , Y_{Cd} , etc. are the calculated values for percentage of amorphous alkaloids, cinchonidine, etc. per bark for any value of quinine (X_Q), cinchonidine (X_{Cd}) or cinchonine (X_C) percentage bark lying within the range of values found for the sections analyzed. Similarly with Y_{Am}' , X_Q' , etc., but these are expressed as a percentage of the total alkaloids. P shows the level of significance of the regression. N.S. not significant.)

A	Tree no 1970	P	Tree no 1512	P
Y_{Am}	$2.006 - 0.198 X_Q$	0.05	$1.754 - 0.091 X_Q$	N.S.
Y_{Am}	$1.796 - 0.640 X_{Cd}$	0.05	$1.602 - 0.206 X_{Cd}$	N.S.
Y_{Am}	$1.466 - 1.419 X_C$	0.30	—	—
Y_{Cd}	$-0.216 + 0.278 X_Q$	0.01	$0.183 + 0.109 X_Q$	0.01
Y_{Cd}	$-0.040 + 0.044 X_Q$	0.10	—	—
Y_C	$-0.034 + 0.196 X_{Cd}$	0.01	—	—
B				
Y_{Am}'	$71.11 - 0.881 X_Q'$	0.01	$48.57 - 0.533 X_Q'$	0.01
Y_{Am}'	$44.48 - 1.910 X_{Cd}'$	0.01	$39.29 - 1.750 X_{Cd}'$	0.10
Y_{Am}'	$27.15 - 2.820 X_C'$	0.10	—	—
Y_{Cd}'	$-4.25 + 0.287 X_Q'$	0.01	$4.023 + 0.088 X_Q'$	0.02
Y_C'	$-0.34 + 0.038 X_Q'$	0.40	—	—
Y_C'	$-0.82 + 0.222 X_{Cd}'$	0.02	—	—

a living branch when the tree was felled. At section 34, where the Q/Cd ratio was relatively high, there were no branches. In the upper half of the tree, where many living branches joined the main stem, there was a marked fluctuation in the Q/Cd ratios. The highest quinine contents were found in the three sections taken from the lowest quarter of the tree, roughly between ground level and 1.5 m.

In the lateral branches there was a rapid fall in the total alkaloid content of the bark from the primaries to the quaternaries from 2.71 to 0.68%. Large scale extractions of the leaves separated into groups ranging from young to mature showed total alkaloid contents ranging from 0.16 to 0.33%, but no correlation was found between leaf age and total alkaloid content. The combined total alkaloids (1 g) from the leaves gave a tartrate equivalent to 0.0156% quinine plus cinchonidine in the leaves, but neither quinine nor cinchonidine could be isolated from the decomposed tartrate.

DISCUSSION

The distribution of the alkaloids in the stem bark is a function of bark thickness. In general the thickness of the bark at any point is inversely proportional to the distance of that point from the base of the tree, but a regular decrease of bark thickness from the base upwards is rare. In these investigations local thickening of the bark was found where the main stem forked, usually caused by damage of the stem at these points at an earlier stage in the tree's history, where secondary leaders arose, probably due to a similar cause, and where large branches joined the stem. Superimposed, therefore, on what is intrinsically a regular decrease in alkaloid content from the base of the tree upwards, are zones of relatively high alkaloid content, the locations of which are governed in each tree by the history of its growth as reflected in its form.

Table 2 shows, for the stem bark as a whole, the increases or decreases in alkaloid content that had been associated with unit increase in bark weight during the life of the tree. Significant increases were found for total alkaloids, quinine and cinchonidine/unit increase in bark weight, and for quinine, cinchonidine and cinchonine/unit increase in total alkaloids. These increases were large compared with the small and insignificant increases (trees 2137 and 1512) and decreases (tree 1970) found for the amorphous alkaloids, an indication of the progressive conversion of amorphous to crystallizable alkaloids during the life of the tree. When the trees were felled the amorphous alkaloid content was that currently available for transformation to the crystallizable alkaloids, while the crystallizable alkaloid content represented the accumulation of the end products of the amorphous alkaloid transformations throughout tree growth.

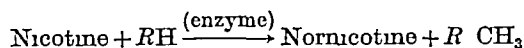
The negative regressions shown in Table 3 lend support to the theory that the amorphous alkaloids are the precursors of the crystallizable alkaloids, additional evidence for which is furnished by observations made here that the bark of young trees, or from the younger upper parts of an old tree, contain a high proportion of amorphous in relation to crystallizable alkaloids.

The proportions in which quinine, cinchonidine and cinchonine are laid down must depend on the conditions obtaining in the bark when the amorphous alkaloids are converted. If the crystallizable alkaloids were formed simultaneously and in definite proportions, their ratios would be constant throughout the bark irrespective of its position on the tree. It was found, however, that the Q/Cd and Q/C ratios were notably low in the root bark and in the bark where the main stem forked, where secondary leaders arose, and where there was abnormal bark thickening. These low ratios do not imply a low quinine content, but rather a relatively greater increase of cinchonidine and cinchonine compared with that of quinine. It is evident that there is a preferential formation of cinchonidine and cinchonine where there is increased cambial activity following some form of injury or other stimulus, further evidence for which is furnished by the almost complete lack of cinchonine in the normally growing tree 1512. These observations, in conjunction with the relationships already established between bark thickness, total alkaloid content and abnormal bark growth, indicate that cambial activity plays a major role in determining the distribution of the total alkaloids in the bark, and the proportions in which the crystallizable alkaloids are laid down. Cambial activity may further govern the efficiency or degree of conversion of the amorphous to the crystallizable alkaloids, for in tree 1970 the highest crystallizable to amorphous alkaloid ratios obtained where the tree bent and where the secondary leader arose.

Botanical investigations in Java (private communication) have shown that a high quinine content is found where vertical growth is stunted and that in such areas *helopeltis* attacks are common, which further restrict growth and increase the quinine content. While a valid comparison of the average percentages of the individual alkaloids in the three trees here investigated is not strictly admissible (since the bark samples were not selected purely at random), it appears from the analytical data that tree damage was associated with an increased percentage of crystallizable alkaloids and a decreased percentage of amorphous alkaloids as compared with those in the normal tree, a further indication of some relationship between cambial activity and the transformation of amorphous to crystallizable alkaloid.

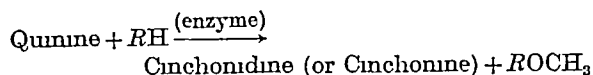
The high percentages of total alkaloids associated with local bark thickening suggests an increased translocation of amorphous alkaloids or their precursors to these places, possibly as soluble salts. The low Q/Cd and Q/C ratios coincident with abnormal thickening, and the variation in the proportions of the individual alkaloids and their ratios from bark section to bark section, indicate that the crystallizable alkaloids, once formed, are not further translocated. Their movement seems to be restricted towards the outer periphery of the bark as it ages and thickens, while further transformations of amorphous to crystallizable alkaloid take place in the newer underlying bark. It is unlikely that the proportions in which the alkaloids are laid down in any particular section of bark remain constant during tree growth. The association between decreasing Q/Cd ratio and increasing height of bark above ground level found in the lower half of the normal tree suggests that, at least under normal conditions of growth, the transformation of amorphous to crystallizable alkaloids shifts progressively in favour of quinine as the bark ages, possibly as a result of a progressive change in cambial activity.

Dawson (1945), in his work on the physiological chemistry of the tobacco alkaloids, has demonstrated that normicotine is produced *in vivo* from nicotine. From a consideration of their respective molecular structures he suggested that nicotine is acted upon in the cell by a transmethylating agent and a methyl acceptor (RH) and proposed as a working basis the hypothetical mechanism



This faculty, he considered, is inheritable.

The simultaneous increase of quinine, cinchonidine and cinchonine in the bark does not preclude the possibility that quinine may at the same time be degraded to cinchonidine and cinchonine, but at a slower rate than its synthesis from the amorphous alkaloids. The transformation may be postulated as



This reaction assumes the presence of a trans methoxylating agent and a methoxyl acceptor, the reaction rate depending on the RH content of the bark, which is primarily governed by the age of the bark and its location on the tree. Such a mechanism could account for the wide fluctuations observed in the Q/Cd and Q/C ratios in the bark of any particular tree and, if inheritable, for the predominance of quinine in *C. Ledgeriana* and cinchonidine in *C. succirubra*.

- SUMMARY

1 Alkaloid distribution in the bark of three trees of *Cinchona Ledgeriana* has been investigated. Two of the trees had suffered damage at an earlier stage of their growth and differed markedly in shape from the third tree which was of apparently normal growth and straight in form.

2 Significant positive regressions were found for percentage total alkaloids on bark weight, thickness and volume. Increases in bark weight and total alkaloids were associated with significant increases in quinine, cinchonidine and cinchonine at the expense of the amorphous alkaloids.

3 Local thickening of the bark was found where the tree forked, where secondary leaders arose and where the tree had been damaged. At these places the alkaloid content was notably high and the quinine/cinchonidine (Q/Cd) and quinine/cinchonine (Q/C) ratios relatively low. Similar conditions obtained in the root bark. Local thickening was less pronounced with the normal tree, the bark of which showed, from the ground upwards, a fairly regular decrease in the percentage of total alkaloids and in the Q/Cd ratios, in contrast to the wide fluctuations observed with the other trees.

4 Meristematic or cambial activity appears to be an important factor in determining the alkaloid content of the bark, the degree of conversion of the amorphous to the crystallizable alkaloids, and the proportions in which the latter are laid down. The distribution of the alkaloids throughout the bark is therefore primarily governed by the history of the tree as reflected in its form.

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The Effect of some Thiol Compounds upon Trypsin, Chymotrypsin and Chymotrypsinogen

By R A PETERS AND R W WAKELIN, *Department of Biochemistry Oxford*

(Received 1 September 1947)

WITH AN ADDENDUM ON

Examination of Chymotrypsin in the Ultracentrifuge

By R CECIL, *Department of Biochemistry, Oxford*

(Received 21 October 1947)

In the course of a research with another objective during the war in 1943-4, we found that thiol containing compounds inactivated trypsin and chymotrypsin. This was not reported then. In the interval, Grob (1945-6) has published work upon trypsin done independently in 1943 and with somewhat similar observations. The work which we have now completed was carried out upon crystalline chymotrypsin and trypsin and with a different criterion for digestion, some observations have been added upon the behaviour of chymotrypsinogen. Our results for trypsin are in substantial agreement with those of Grob, though our interpretation of the phenomenon is different.

EXPERIMENTAL

Trypsin and chymotrypsin were prepared from ox pancreas by the methods of Kunitz & Northrop (1935-6) and stored in a desiccator at 2°. The trypsin used was crystalline, but had not been recrystallized; it contained 32.6% enzyme protein, accepting the usual figure for the N content of trypsin as 16%. The chymotrypsin had been once recrystallized and had a protein content of 73.7% (N, 15.5%). Ultracentrifugal examination by Mr R Cecil (see Addendum) showed that the specimen was homogeneous. The chymotrypsinogen investigated had been reprecipitated (protein content 90.2%). It was part of the specimen used in preparation of chymotrypsin; no attempt was made to separate different forms of chymotrypsin (Kunitz, 1938-9). The activity of both enzymes was estimated by determining the extent of digestion of casein using micro Kjeldahl estimations of N not precipitable by trichloroacetic acid (N.P.A.).

For preparation of the casein substrate the method essentially followed that of Northrop & Kunitz (1932-3). Casein (Glaxo A/E, 50 g) was stirred into 0.1M phosphate buffer, pH 7.6 (11), approx. 5N NaOH (3-3.5 ml) was added and the mixture heated on the water bath for 30 min with additions of 5N NaOH to maintain the pH at 7.6. After cooling and removal of undissolved casein by centrifuging, the solution was kept in the refrigerator, thymol being added. Values for the preformed N.P.A. varied from 1 to 2 mg./6 ml. casein substrate; different batches of casein substrate showed variations in digestibility.

Experiments were in all cases planned to show differences due to the action of added thiol compounds. Each was set up with a control, weighed sample of the same enzyme. As no attempt was made to homogenize the enzyme preparation, this procedure controlled possible variations in the salt content of samples taken. Usually the enzyme (1-2 mg) was incubated in 0.1M phosphate buffer, pH 7.6 (1.0 ml), for 45 min, after which 6 ml. casein substrate were added. After digestion for 40-50 min at 38°, 1 ml. 25% (w/v) trichloroacetic acid was added, after standing for 30 min at room temperature the precipitate was filtered and washed twice with 5 ml. 2.5% (w/v) trichloroacetic acid. After adjusting the volume to 25 ml, 5 ml. were taken for ashing by micro-Kjeldahl. Most incubations were done in narrow test tubes in air, results in N₂ were not appreciably different. Glass distilled water was used to reduce contamination by metals to a minimum.

In the earlier experiments the thiol compound was added directly to the digestion mixture, all later experiments were done by a previous incubation of thiol with the enzyme before addition to the casein. The results were qualitatively similar, but the latter procedure gave a much more marked effect. Several experiments were made with crude trypsin (liquor pancreaticus, Bengel) which are not included, as they are difficult to interpret (see below).

The results are expressed as the difference in N.P.A. determined by the micro Kjeldahl method in the digests with untreated enzyme and with enzyme incubated with the substance under test, the usual unit being mg. N.P.A./6 ml. casein substrate. In the experiments of 1943-4, control values after digestion with 1 mg. chymotrypsin were usually 14-15 mg. N.P.A., and with 2 mg. trypsin approx. 8 mg. N.P.A. For trypsin a control was done with the specific substrate, N⁷ benzoylarginine amide.

Control experiments with suitable samples to which no thiol was added showed that during the periods of incubation less than 10% of trypsin or 5% of chymotrypsin was inactivated. There was no evidence that SH compounds had an appreciable effect on the course of digestion; the thiol concentration during digestion was one seventh of that in the incubation mixture, i.e. incubation with 0.1M thiol was followed by digestion at 0.014M, the rate of inactivation is lower for the enzyme in absence of substrate at this reduced concentration of thiol (see Fig. 1), and with casein present the rate is still lower, and not more than 1-2% extra

inhibition was found in a 30 min digestion period. If the concentration of thiol was maintained at 0.1M throughout the incubation and digestion periods by addition of extra MTEG with the casein, it was found that extra inhibition occurred, not exceeding 10%, interpreted as due to further inactivation by thiol of the enzyme itself. In one experiment upon trypsin, in which the thiol was removed by oxidation prior to digestion, the results were in agreement with this view. Hence we have no evidence that thiol compounds were inhibiting the course of digestion in these experiments, and it seems logical, therefore, to describe our effects as due to inactivation.

RESULTS

Inactivation of the proteinase activity of trypsin or chymotrypsin, or of both, has been shown by the following SH compounds: monothioethyleneglycol (MTEG), cysteine ester hydrochloride, glutathione,

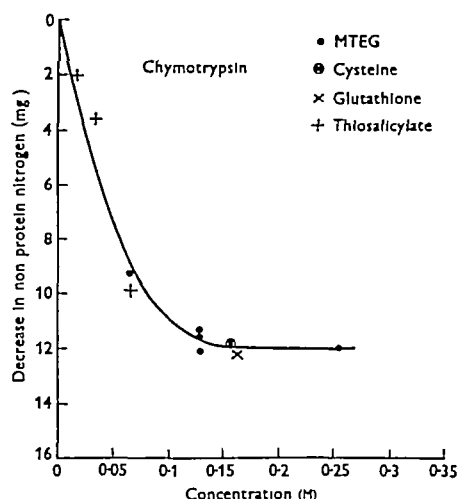


Fig 1 Effect of previous exposure to varying concentrations of thiol compounds on the digestion of casein substrate by crystalline chymotrypsin. Addition of 1 mg enzyme preparation to 6 ml. casein solution, pH 7.6, 38°. Digestion time 45 min. Results expressed as decrease in mg of NPN formed.

2,3-dimercaptopropanol (BAL), sodium sulphide and thioallicylic acid, slight inactivation (14%) was produced by sodium cyanide (0.2M) at pH 7.8, no inactivation was produced by oxidized monothioethyleneglycol (0.25M), cystine ester hydrochloride (0.07M), thiosulphate (0.2M on chymotrypsin), sodium diethyldithiocarbamate (0.09M) or pyrophosphate (see below), also, in a different category, no inactivation was produced by lewisite oxide (0.65 mM), allantoin (0.038M), cetyltrimethylammonium bromide (CTAB) (1 mg/ml) or heparin (1 mg/ml). MTEG and cysteine also had a special action upon chymotrypsinogen.

Figs 1 and 2 show in detail the effect of increasing molar concentrations of some thiol compounds acting for 45 min at 38° upon chymotrypsin and trypsin. At approx 0.1M under these conditions,

there was an almost maximum inactivation. The degree of inactivation induced was approximately the same for each thiol compound, this suggests that the thiol group is the active agent.

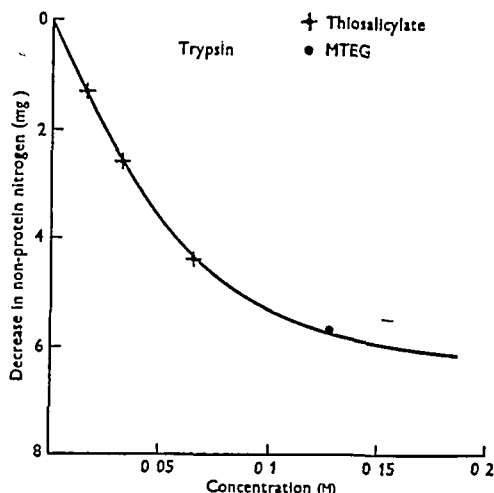


Fig 2 Effect of previous exposure to thiol compounds on the digestion of casein substrate by crystalline trypsin. Addition of 2 mg enzyme preparation to 6 ml casein solution, pH 7.6, 38°. Digestion time 45 min. Results expressed as a decrease in mg of NPN formed.

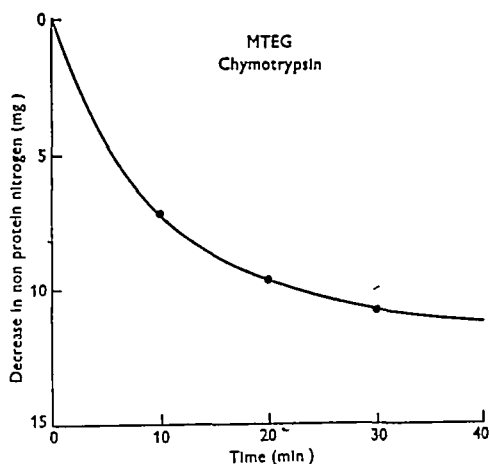


Fig 3 Effect of time upon the inactivation of chymotrypsin activity by 0.127M monothioethyleneglycol. Casein digest, pH 7.6. Results expressed as decrease in mg of NPN formed.

With sodium sulphide on chymotrypsin the results varied much with the alkalinity, working in tightly stoppered vessels to avoid escape of hydrogen sulphide and at pH 7.6, decreases in NPN with 1 mg chymotrypsin were observed as follows: for 0.082M sulphide, 4.05 mg and for 0.125M, 5.5 mg NPN.

The relation between inactivation of 1 mg chymotrypsin due to 0.127M MTEG and the time of action is shown in Fig 3, and with this concentration the action was rapid.

The effect of varying the pH with chymotrypsin and MTEG is shown in Fig 4, where it is clear that the action was much increased as the pH rises above 7.0, this would be expected in the case of a thiol effect

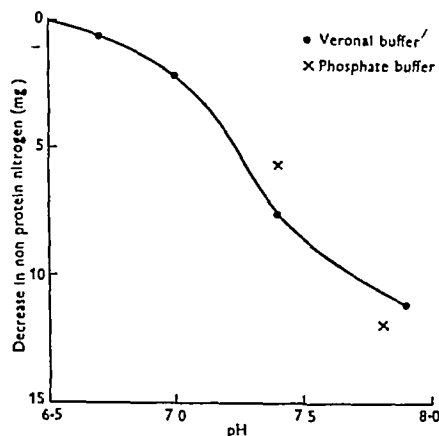


Fig 4 Effect of varying pH on the inactivation of chymotrypsin activity by 0.127M monothioethyleneglycol. Digestion time 45 min for 1 mg enzyme added to 5 ml casein solution, pH 7.6. Results expressed as decrease in mg N.P.N. formed

The inactivating effect of thiols upon the action of trypsin upon the specific substrate N^1 benzoyl-arginine amide (Bergmann, Fruton & Pollok, 1930) was also proved in two experiments, of which one is quoted in Table 1

Table 1 Inactivating effect of monothioethyleneglycol (MTEG) and 2,3 dimercaptopropanol (BAL) upon the digestion of N^1 -benzoylarginine amide

(Volumes of 0.01N ethanolic HCl required by 0.5 ml. substrate titrated in 90% acetone (Landerstrom Lang technique) before and after digestion at 38° for 2 hr and containing initially 0.051M benzoylarginine amide and 0.4 mg trypsin)

Addition	Start (ml.)	2 hr (ml.)	Diff (ml.)
None	+0.08	+1.27	+1.19
MTEG (0.128M)	+0.06	+0.05	-0.01
BAL (0.031M)	+0.43	+0.51	+0.08

Table 3 Attempted reactivation of chymotrypsin (1 mg) and trypsin (2 mg) with cystine ester or oxidized glutathione, respectively, after inactivation by sodium sulphide or monothioethyleneglycol (MTEG)

	Inactivating compound	Attempted reactivation by	Decrease in N.P.N. found (mg)
(a) Chymotrypsin	0.082M Na_2S	—	4.05
	0.125M Na_2S	—	5.5
	0.082M Na_2S	0.29M cystine ester*	5.1
	0.125M Na_2S	0.29M cystine ester*	6.4
	0.64M MTEG	—	3.25
(b) Trypsin	0.64M MTEG	0.13M oxidized glutathione	3.25

* Reckoned on S content

These phenomena were so much like those already well known for insulin (cf Abel & Geiling, 1925, du Vigneaud, 1927), where reduction of a S-S group leads to inactivation, that they led to attempts to get evidence (i) that S-S compounds had no effect on these enzymes, (ii) that there was no reactivation upon reoxidation with S-S compounds, and (iii) that a SH group was present after treatment with the thiol. The results were as follows

(i) Neither cystine ester hydrochloride (30 mg/ml) nor oxidized MTEG (20 mg/ml) produced any effect (Table 2)

Table 2 Effect of oxidized MTEG, and of cystine ester HCl upon the activity of chymotrypsin

(All results expressed for 1 mg enzyme in 1 ml added to 6 ml casein solution. Inhibitors added at start of digestion, 38° for 45 min)

Addition	N.P.N. formed (mg)	Decrease in N.P.N. formed (mg)
None	14.0	—
0.256M MTEG	—	4.5
0.256M MTEG (oxidized)	—	0.15
0.127M Cystine HCl	—	2.8*
0.162M Cystine ester HCl	—	0.4*

* In arriving at these figures, a blank has been subtracted for the N present in the cystine or cysteine

(ii) An attempt to reactivate chymotrypsin and trypsin, after exposure to sodium sulphide and MTEG and subsequent removal of unchanged thiol, is shown in Table 3 (a) and (b), relevant details of the experiments are given below. No reactivation was found either with cystine (as ester) or with oxidized glutathione

The treatment of chymotrypsin in (a) was as follows. After exposure for 45 min to the sulphide at pH 7.6 in a stoppered tube to prevent escape of H_2S the unchanged H_2S was removed *in vacuo* at pH 6.0 (HCl) until none was detectable by smell. Cystine ester (0.29M, reckoned on S content) was added, the pH readjusted, and the whole incubated for a further 45 min. Casein substrate (6 ml.) was added and digestion carried on for 45 min. Trichloroacetic acid (25% w/v, 1 ml) was then added, and the whole left to stand for 30 min. The precipitates were washed three times

with 2.5% trichloroacetic acid (5 ml) and the filtrates made up to 25 ml, 5 ml. being taken for analysis. The precipitates were left standing with 5% trichloroacetic acid (5 ml) for 18 hr, after which the supernatant fluid gave no nitroprusside test for cystine, indicating complete extraction. The control enzyme was incubated for 1.5 hr, NaCl (12 mg/ml) being added equivalent to that formed from Na_2S in the experimental tube.

The treatment of trypsin in (b) was as follows. After exposure to MTEG for 2 hr and cooling, the solution of inactivated enzyme was saturated with MgSO_4 and allowed to stand in the cold. The precipitate was removed by a small filter, washed with saturated MgSO_4 until the washings were free from SH, and then taken up in phosphate buffer, pH 7.6. A sample was removed for estimation of activity, and the remainder treated with oxidized glutathione and incubated for 2 hr at 38°. Further samples were then taken to estimate the activity.

(iii) Evidence of the appearance of SH groups was sought by titrating the enzymes with porphyrindin solution (Kuhn & Desnuelle, 1938) before and after treatment with monothioethyleneglycol. Mirsky & Anson (1934-5) used thioglycolic acid, but MTEG has the advantage that no COOH group is present to form possible salts. Some difficulty was experienced in making certain that any effect observed was not due to traces of MTEG absorbed on the precipitates or filter papers. After treatment with the thiol, trypsin was clearly much altered, it was no longer precipitable by 5% trichloroacetic acid, which prevented use of this reagent in washing. Two procedures were finally adopted, viz precipitation by ammonium sulphate, and treatment with 90% acetone (Stern & White, 1937), both at acid reaction, to minimize oxidation of any thiol group. Examples of these are given below, Exp 1 for chymotrypsin, Exp 2 for trypsin and Exp 3 for the parent protein chymotrypsinogen.

In these experiments, the ratio of thiol to enzyme was reduced for experimental convenience. The relation of thiol concentration to rate of inactivation has not been systematically investigated, but control experiments showed that the concentrations used in the experiments below were adequate to inactivate substantially within 30 min.

Exp 1 (7 viii 47) Chymotrypsin (50 mg containing 36.8 mg ash free enzyme) was dissolved in 2 ml 0.05M phosphate buffer, pH 7.6, 100 mg MTEG were added and the pH again adjusted. After incubation at 38° for 120 min, the mixture was cooled, N HCl was added to bring the pH to 3.0, and saturated $(\text{NH}_4)_2\text{SO}_4$ added to 60% of saturation. The precipitate was removed by centrifuging (2500 r.p.m.) and then transferred to a very small pressure filter (filter paper 8 mm diam), the precipitate was washed on the filter with saturated $(\text{NH}_4)_2\text{SO}_4$ solution until the filtrate gave no nitroprusside reaction for thiol, it was then suspended, with the paper, in 0.001N HCl, reprecipitated with $(\text{NH}_4)_2\text{SO}_4$ and again washed on a fresh filter paper. This operation was repeated once again. The washed precipitate of 'thiol treated' enzyme was then suspended in phosphate buffer,

pH 7.3, and immediately titrated with porphyrindin (0.5 mg/ml standardized against cysteine ester hydrochloride) until fresh additions of 0.025 ml. did not decolorize in 30 sec. The total amount used was 1.05 ml porphyrindin, equivalent to 0.69 mg cysteine ester HCl. One earlier experiment gave 87% of this value.

Reckoning the S content of chymotrypsin as 1.85% (Kunitz & Northrop, 1934-5), this means that approximately one fifth (17.4%) of S had appeared as SH groups. In a control experiment run at the same time, a solution of 25 mg of chymotrypsin, not treated with thiol during the incubation, was acidified, MTEG was then added, and the protein immediately precipitated with $(\text{NH}_4)_2\text{SO}_4$ and the washing procedure carried out as above. When dissolved in phosphate buffer this control chymotrypsin reduced less than 0.025 ml porphyrindin solution.

In another experiment with chymotrypsin and MTEG done by the method described under Exp 3, the chymotrypsin washed free from MTEG gave a marked nitroprusside reaction for SH upon addition of nitroprusside and ammonia.

Exp 2 (9 viii 47) Trypsin (50 mg) was treated with 2.0 ml phosphate buffer, pH 7.8, the precipitated magnesium phosphate was removed by centrifuge, and the resulting solution placed in a closed vessel (Stern & White, 1937), N_2 was passed at 38°. After 5 min MTEG (100 mg) was quickly added, the resultant pH was 7.6. After action for 60 min. in the N_2 atmosphere, 0.3 ml. N HCl was added, the mixture cooled and 9 vol. of acidified acetone (0.55 ml N HCl to 250 ml) added. After centrifuging, the precipitated protein was washed 6 times with the acidified acetone and dried in the centrifuge tube *in vacuo*. There was no smell of thiol. The protein was dissolved in 1 ml phosphate buffer, pH 7.3, and immediately titrated with porphyrindin, using an amount of dye equivalent to 0.106 mg cysteine ester hydrochloride. Reckoning 1.1% as the S content of trypsin (Kunitz & Northrop, 1934-5) this gives 10.2% of S reduced, a value which should be increased by at least one tenth to allow for losses during the initial washing.

A previous experiment with trypsin, carried out on the lines of Exp 1 with $(\text{NH}_4)_2\text{SO}_4$ fractionation, gave 8.2% of S reduced, which is of the same order as that in Exp 2. Trypsin (50 mg), not treated with thiol until the washing stage, reduced less than 0.02 ml dye.

Exp 3 (20 viii 47) Chymotrypsinogen (50 mg) was treated with 2.0 ml phosphate buffer, pH 7.8, and MTEG (100 mg). Rapid precipitation took place, most of the protein coming out of solution in 10 min. After 30 min at 38°, the solution was cooled on ice, the precipitated protein was removed by centrifuging, and suspended in 0.02N HCl (2.0 ml), 0.2 ml. N HCl was added and 9 vol. of cold acidified acetone. After removing the precipitate by centrifuge, it was resuspended in cold 0.02N HCl, 9 vol. of acid acetone added, and the precipitate washed with acetone, the resuspension and washing were repeated, and the precipitate was finally dried *in vacuo*. There was no smell of thiol. The dried product was suspended in phosphate buffer, pH 7.3 (1.0 ml), transferred to a small test tube and titrated with porphyrindin until small additions were not decolorized in 30 sec, the porphyrindin used was equivalent to 0.397 mg cysteine ester HCl. Reckoning chymotrypsinogen S as 1.48% (Brand & Kassell, 1941-2), this corresponds to a reduction of 15.4% of the S present. Before thiol treatment, chymotrypsinogen showed no SH content by porphyrindin titration.

These experiments have convinced us that reducing thiol groups are formed in these proteins by the action of MTEG, they accounted for one tenth to one fifth of the total S stated to be present, but the exact amounts of thiol which can be formed must be decided by future, more accurate experiments. In this connexion Brand & Kassell (1941-2), in a very pure specimen of chymotrypsinogen, have found the content of methionine S to be 0.262% and of cystine + cysteine S, 1.225%, it is not known how the S content changes upon conversion to chymotrypsin, but they record the odd point that hydrolysis with hydrochloric acid yields 4 cysteine groups/molecule, they state that no reactive SH was present in chymotrypsinogen as determined by nitroprusside and ammonia, with which our titration values agree.

Relation to denaturation The appearance of thiol groups in these experiments is not similar to the well known unmasking of thiol groups in the denaturation of egg albumin, in the controls mentioned, which had been treated with acid acetone, the chymotrypsin and chymotrypsinogen were denatured in the sense that the protein became insoluble, without liberation of thiol. Similarly, neither boiling the three proteins in water to which a trace of acetic acid had been added to slow down oxidation of any possibly liberated thiol, nor treatment with purified guanidine hydrochloride (6M) (Greenstein, 1938) led to a liberation of thiol, subsequent addition of sodium nitroprusside and ammonia gave no thiol reaction, which readily appeared upon addition of minimal amounts of cyanide. The denatured proteins had a disulphide grouping and behaved exactly as denatured serum albumin (Walker, 1925).

All the evidence so far available suggests that the behaviour of trypsin and of chymotrypsin, as well as of chymotrypsinogen, to treatment with thiols is similar to that of insulin.

Attempted inactivation by pyrophosphate In view of the statement by Grassmann, Dyckerhoff & Schoenebeck (1930) that trypsin kinase was inactivated by pyrophosphate as well as by cysteine, this

has been investigated. No effect was found in two experiments, details of one are given in Table 4.

Table 4 *Effect of pyrophosphate (pH 7.6) upon activity of crystalline trypsin (2 mg)*

(Incubated for 3 hr. with and without sodium pyrophosphate (0.224M), casein substrate then added and digestion carried out for 40 min.)

	Nitrogen formed (mg)
Control	3.80
Pyrophosphate	4.00

Relation to metals As might be expected, there was no change in the degree of inactivation due to thiol produced in our experiments by addition of manganese or zinc (1 mg) together with the thiol. The action of thiols was not an effect on metals. The activation of papain by cyanide and thiol compounds is well known, and also the pioneer experiments of Hopkins & Morgan (1938) upon the reversible inactivation of succinate dehydrogenase by S-S compounds. As noted by Grob (1945-6), the only earlier reference to an inactivating action of thiol compounds upon trypsin, by Grassmann *et al.* (1930), referred to inactivation of trypsin kinase at pH 8.9 in 0.2M ammonia ammonium chloride buffer solution. Using amounts of cysteine up to 0.02M, it was found that trypsin alone was substantially inactivated, after adding intermediate amounts of kinase, the extra digestion due to kinase was inhibited, even by 0.001M cysteine, addition of extra kinase practically abolished the inhibition. Pyrophosphate behaved in a similar way. It is to be noted that they were using an ethanolic titration as a method of estimation. Since we found no effect of pyrophosphate, in our opinion their results would be better interpreted as being due to the action of a peptidase, present in the trypsin preparation, which was activated by some divalent metal added with the kinase. It might be expected that the effect of the metal would be removed by combination with a thiol or with pyrophosphate.

We have tried the effect of adding thiol upon the manganese activation of the peptidase which splits

Table 5 *Increasing effect of 0.288M MTEG upon the activation by manganese of aminopeptidase in a Fruton type extract of rat skin as the manganese concentration is reduced. Substrate, L-leucylglycine*

(Samples of 0.5 ml, containing 4.67 mg L-leucylglycine and 0.2 ml skin extract, taken after digestion for titration in 90% acetone by the Linderstrom-Lang technique. Results given are increases in 0.01N-ethanolic HCl titrated as a result of the digestion.)

Exp	Digestion time (38°) (hr)	Addendum	Manganese addition		
			Ni (ml)	0.0037M (ml)	0.00037M (ml)
4	3.5	Ni	0.33	2.16	—
		MTEG	0.23	1.63	—
5	2	Ni	0.38	—	1.85
		MTEG	0.01	—	1.00

L-leucylglycine in skin (Fruton, 1946, Neville Jones & Peters, 1948), and have found a decreasing effect with the addition of larger amounts of manganese, a result much in keeping with the results of Grassmann *et al* (Table 5)

DISCUSSION

Using smaller amounts of crystalline trypsin than in these experiments Grob found with approx 0.01M cysteine, 59% inhibition, with 0.04M sodium thioglycollate, 26%, with 0.003M glutathione, 17%, and with one fifth saturated H_2S solution, 7%. These inhibitions are less complete than those noted by us. He also found a 50% inactivation with 0.05M cyanide. Working at pH 7.6–7.8 we have only found slight inhibition with 0.2M cyanide. It is to be noted that Blatherwick, Bischoff, Maxwell, Berger & Sahyun (1927) found that, at pH 7.0, 0.4M cyanide took 18 hr to inactivate insulin completely. Grob considered that his results showed a sensitivity to oxidation reduction in the protease molecule, since we have found that there is no reactivation by exposure to S-S compounds and that the molecule seems definitely to come to pieces, we think the phenomenon is irreversible. The precipitation of chymotrypsinogen by SH action is the first case known to us in which such a change of state can be produced by reduction alone, in this it seems uniquely interesting.

It is interesting also that at least three active proteins from the pancreas are inactivated by reduction, in addition to the similar action on chymotrypsinogen. It is natural to think that inactivation by reduction may have biological importance, but

for the present we have no evidence of this and must point out that the concentrations of thiol agents used here were relatively very high.

Note added 21 October 1947 Since the completion of this manuscript our attention has been drawn to the statement by E. C. Webb & R. van Heyningen that 0.0042M-BAL had no effect upon a commercial specimen of trypsin (Webb & van Heyningen, 1947). The result does not conflict with our statements, as the concentration used was relatively small and the time of exposure to the enzyme alone not more than 15 min.

SUMMARY

1 Several thiol compounds, monothioethylene glycol, cysteine, glutathione, 2,3-dimercaptopropanol, thioalicyclic acid and sodium sulphide irreversibly inactivate crystalline trypsin and chymotrypsin.

2 This action, like the well known effect on insulin, increases on the alkaline side of the neutral point.

3 The inactivating effect is not reversible by exposure to S-S compounds, and is accompanied by the appearance of SH groups. This is not an unmasking of thiol groups by ordinary denaturation.

4 SH groups also appear in chymotrypsinogen upon treatment with some thiol compounds, and the protein is precipitated.

Our thanks are due to Mr A. Harrison for help with the preparation of the crystalline enzymes, to Dr L. A. Stocken for cystine and cysteine ester hydrochloride and for porphyrin, and to Dr Whittaker for monothioethyleneglycol.

ADDENDUM

Examination of Chymotrypsin in the Ultracentrifuge

By R. CECIL, *Department of Biochemistry, Oxford*

A sample of chymotrypsin was examined in the Svedberg oil turbine ultracentrifuge by the method of Philpot (1938). The concentration of protein was 0.74% (refractive increment at $546 m\mu = 0.00134$) in buffer containing 0.2M-NaCl, 0.058M KH_2PO_4 , and 0.008M- Na_2HPO_4 . The speed was 1020 rev/sec.

The sedimentative diagram (Fig. 5) showed a single homogeneous component of $S_{20}(\text{corr}) = 3.09 \times 10^{-13}$. Integration of the area of the boundary accounted for 75% of the total refracting material.

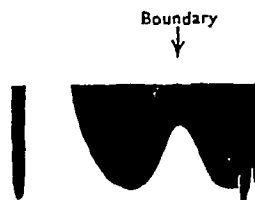


Fig. 5. Sedimentative diagram of chymotrypsin, showing the boundary 70 min. after reaching full speed.

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Quantitative Determination of Glutamine and Glutamic Acid

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(Received 23 October 1947)

Washed suspensions of *Clostridium welchii*, strain S R 12, as shown by Gale (1945, 1947), specifically decarboxylate L glutamic acid and L glutamine and are thus a specific reagent for the determination of these two substances. It is shown in this paper that the decarboxylation of glutamine is accompanied by the formation of an equivalent amount of ammonia, and that the glutaminase responsible for this reaction is highly specific. No other compound has so far been found to yield ammonia when incubated with washed suspensions of the organism at pH 4.9. This glutaminase preparation is more convenient to make than Archibald's (1944) glutaminase preparation from kidney and is more specific.

The following method for the quantitative determination of glutamic acid and glutamine rests on these facts, and combines the principles used by Gale (1945) and by Archibald (1944). The sum of the substances is determined according to Gale's procedure, slightly modified, by manometric determination of the carbon dioxide evolved on decarboxylation. The ammonia is then estimated in the solution treated with *Cl. welchii*, and in a sample not so treated. The difference represents the glutamine.

Preliminary experiments indicated that the rate of CO₂ evolution from glutamine is under some conditions considerably slower than the CO₂ evolution from glutamic acid, and a detailed study of the conditions favourable for the quantitative decarboxylation of glutamine was therefore required.

EXPERIMENTAL

Bacteria. The strain S R 12 of *Cl. welchii* as recommended by Gale (1945) was used for most experiments. In a few cases, a strain of the same species supplied by the Department of Bacteriology of this University and containing a histidine and a glutamic decarboxylase was used. The organisms were grown at 38° in the medium suggested by Gale except that 50 ml of yeast extract were added to 1 l medium (prepared by autoclaving baker's yeast with an equal volume of water and filtering). The washed cells from about 1 l medium were suspended in about 15 ml of 0.45% (w/v) NaCl. The dry weight of the bacteria in the suspension was determined in a 0.2 ml sample. The yield from 1 l of medium was 1.1-1.5 g of dry bacteria. All data on bacterial weights in this paper refer to dry weight. The suspension was kept in a refrigerator where its activity did not appreciably deteriorate within a month.

Chemical determinations. CO₂ was measured manometrically with air in the gas space of the vessels, NH₃ according to the method of Parnas & Heller (see Parnas, 1934).

Chemicals. The L-glutamine used was prepared from mangel wurzels. In spite of frequent recrystallizations from ethanol-water and water, its purity was only 93% as judged by amide N content and CO₂ evolution. Among the impurities were about 1.3% arginine (see Archibald, 1945). The L-glutamic acid preparation was 96% pure. The impurity was probably its optical enantiomorph, as the amino N content agreed with the theoretical value whilst Gale's method yielded 96%.

Table 1 Rate of CO₂ evolution from glutamine and glutamic acid by *Clostridium welchii* at varying substrate concentrations

(Each cup contained 2 ml substrate solution, 1 ml 0.2N acetate buffer (pH 4.9), 0.05 ml bacterial suspension (≈ 11 mg), 30°)

Substrate	Glutamine				Glutamic acid			
Final substrate concentration (M)	0.013	0.0065	0.00325	0.001625	0.013	0.0065	0.0034	0.001625
Total amount of substrate/cup (μ l)	448	224	112	56	448	224	112	56
CO ₂ (μ l) evolved after								
10 min	19	12	6.5	1	34	30	17.5	13
20 min	30	28	14	3	65	55	36	23.5
40 min	91	58	29.5	7	122	120	65	46

Table 2 Effect of pH on the rate of CO₂ evolution from glutamine and glutamic acid by *Clostridium welchii*

(Each cup contained 3 ml 0.1N acetate or lactate buffer, 1 ml 0.01M substrate, 0.5 ml bacterial suspension (11 mg), 30°)

Substrate	Glutamic acid							Glutamine						
Buffer	Acetate					Lactate		Acetate					Lactate	
pH	5.0	4.7	4.4	4.1	3.7	3.4	3.0	5.0	4.7	4.4	4.1	3.7	3.4	3.0
CO ₂ (μl) evolved after														
5 min	60	64	74	74	75	54	34	26.5	21.5	15	10.5	8.5	2.1	1
10 min	154	160	168	166	143	115	74	72	57	35	22	18	5	2
15 min	189	198	203	203	179	170	110	110	109	52	30	25.5	9	3
50 min	211	211	216	218	208	218	203	190	184	132	82	64.5	26	12

Metabolism of glutamine in *Clostridium welchii*

Rate of CO₂ evolution from glutamine Data comparing the rates of CO₂ evolution from glutamine, and from glutamic acid under the same conditions, are recorded in Table 1. Differences in the rates are especially marked at low concentrations of the substrates. As will be shown below, these results, obtained on pure solutions of the substrates, can be modified by the presence of other substances.

Effect of pH Glutamic acid decarboxylase shows only small changes in activity between pH 3.4 and 5.0 (Table 2, see also Gale, 1941). The optimum is fairly broad and extends from about pH 3.7 to 4.4. At pH 3.0 (lactate buffer) the activity is still about 50% of the optimal activity. In contrast, CO₂ evolution from glutamine falls fairly rapidly with pH between 5.0 and 3.0, at pH 3.0, the rate is only 3% of that observed at pH 5.0.

Production of ammonia Fig. 1 shows that the evolution of CO₂ from glutamine is accompanied by the production of ammonia. During the early stages of the reaction more ammonia than CO₂ is formed, but later the yields of both substances become identical. This indicates that the formation of ammonia precedes decarboxylation, as is borne out by the observation that the CO₂ evolution from glutamine, unlike that from glutamic acid, has a short lag period during which the rate of decarboxylation gradually rises (Table 2, Fig. 1). This is to be expected if the substrate of decarboxylation, glutamic acid, is not present at the start, but is gradually formed under the influence of glutaminase. Further

evidence of the primary hydrolysis of the amide group is afforded by inhibitor experiments. Cyanide (3×10^{-3} M), which completely inhibits decarboxylation (Gale, 1945), but has no effect on the evolution

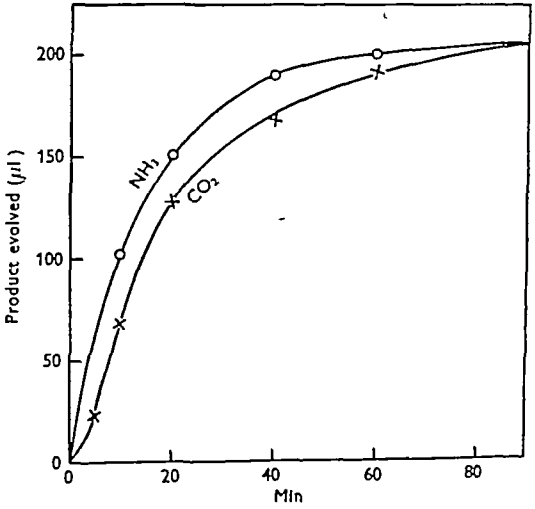


Fig. 1 Formation of NH₃ and CO₂ from glutamine in the presence of *Clostridium welchii*. 3 ml 0.2N acetate buffer, pH 4.9, 1 ml 0.01M glutamine, 0.5 ml bacterial suspension (≈ 11 mg), 30°. The reaction was interrupted by addition of 1 ml 2N HCl at the specified time.

of ammonia, separates the action of glutaminase and glutamic decarboxylase, whilst HgCl₂ inhibits both reactions (Table 3).

Table 3 *Effect of HCN and HgCl₂ on the action of Clostridium welchii on glutamine*

(pH 4.5, 1.40 mg glutamine, 30°)

	Inhibitor		
	None	$3 \times 10^{-3} M$ HgCl ₂	$3 \times 10^{-3} M$ HCN
CO ₂ after 60 min (μl)	171	2	3
NH ₃ after 60 min (μl)	183	~Nil	190

Temperature coefficient of decarboxylation The rate of decarboxylation of glutamine and of glutamic acid at 40° was about 60 % faster than at 30°. There was no indication that the bacterial cells suffered damage through prolonged incubation at 40°.

Effect of narcotics Gale (1946) has already noted that ethylurethane does not inhibit bacterial decarboxylases. Other unspecific 'narcotic' inhibitors like octanol or phenylurethane (both in saturated solutions) were found not to affect the decarboxylation of glutamine.

Salts At pH 4.9 no difference in the rates of decarboxylation was found when the concentration of the acetate buffer was varied between 0.05 and 0.2 M. Higher buffer concentrations caused inhibitions, c. 40 % by 0.4 M buffer and c. 60 % by 0.8 M buffer. NaCl, in presence of 0.2 M acetate buffer, had no appreciable effect as long as its concentration did not exceed 1 M (see also Taylor & Gale, 1945).

Effect of detergents In the course of experiments on inhibitors it was observed that addition of cetyltrimethylammonium bromide ('cetavlon') accelerated the decarboxylation of glutamine. Examples are shown in Table 4. The magnitude of the effect depends, among other factors, on pH. Under the

conditions shown in Table 4, the increase of the initial rate was c. 2.5 fold at pH 4.9, c. 10 fold at pH 4.1 and c. 20 fold at pH 3.8. Thus the shape of the pH activity curve of the decarboxylation of glutamine is changed by the detergent, and the curve obtained with washed cell suspensions is therefore not the true pH activity curve. Other factors, presumably the permeability of the cells, affect the rate of interaction between enzyme and substrate.

Table 5, giving the effects of varying concentrations of cetavlon, shows that 0.48 mg detergent can appreciably increase the activity of 6 mg bacteria and that the maximum effect is produced by about 1 mg detergent.

The decarboxylation of glutamic acid was, under the same conditions, also accelerated by cetavlon, but owing to the relatively high rate of the decarboxylation of glutamic acid without detergent the percentage increase produced by cetavlon was smaller than in the case of glutamine. The effect of adding the detergent to the bacteria before the substrate, e.g. incubation with cetavlon for 20 min before the addition of the substrate, was to produce inactivation (see also Baker, Harrison & Miller, 1941).

An anionic detergent, sodium dodecyl sulphate, completely inhibited the decarboxylation of glutamine under the conditions stated in Table 4, when 0.05 % detergent was added to the substrate solution.

In experiments with another strain of *Cl. welchii*, which decarboxylated histidine in addition to glutamic acid and glutamine, cetavlon likewise accelerated the glutamic decarboxylase but inhibited the histidine decarboxylase, whilst sodium dodecyl sulphate inhibited the decarboxylation of glutamic

Table 4 *Effect of cetavlon on the decarboxylation of glutamine at different pH*

(1.46 mg glutamine in 3 ml. 0.2 M buffer, 40°, 0.1 ml bacterial suspension (3 mg bacteria), cetavlon added to substrate solution)

Buffer		Acetate	Acetate	Lactate	Acetate	Acetate	Lactate
pH		4.9	4.1	3.8	4.9	4.1	3.8
Cetavlon (%)		0	0	0	0.13	0.13	0.13
CO ₂ (μl) evolved after	5 min	26	6	2	68	55	39
	10 min	52	11	4	137	111	86
	15 min	77	13	6	174	172	125
	20 min	96	16	7	187	192	161
	30 min	126	21	8	195	204	186

Table 5 *Effect of varying cetavlon concentrations on the decarboxylation of glutamine*

(1.46 mg glutamine in 4 ml. 0.1 M acetate buffer, pH 4.1, 0.2 ml bacterial suspension (6 mg), cetavlon added to substrate solution)

Cetavlon concentration (final) (%)	0	0.012	0.024	0.048	0.096	2.0
Total amount of cetavlon added (mg)	0	0.48	0.96	1.92	3.84	80
CO ₂ (μl) evolved after						
5 min	2	17	89	78	87	85
10 min	8	44	176	182	181	179
15 min	13	65	201	205	202	205

acid and glutamine and had no effect on the histidine decarboxylase. These experiments will be reported in full elsewhere.

Effect of serum Blood serum (Table 6) also accelerated the decarboxylation of glutamine by *Cl. welchii*, the effect depending on pH. At pH 4.1, larger (c. 10 fold) amounts of serum were required than of cetavlon (on a w/w basis) and the maximum effects of serum were smaller. Gelatin (0.6%) had no effect, 'Bacto' peptone (0.6%) caused a small increase (about 30%) of the initial rate. When both cetavlon and serum were added, the effects observed were somewhat smaller than those of cetavlon alone (Table 6). At pH 4.9, the maximum effects produced by cetavlon and by serum were about equal, and smaller quantities, about one half, were required for the maximum acceleration. Aqueous extracts of animal tissues had effects similar to those of serum.

Table 6 *Effect of serum on the decarboxylation of glutamine at pH 4.1*

(1.46 mg glutamine in 4 ml total volume, 0.1 M acetate buffer, pH 4.1, 40°, 0.1 ml bacterial suspension (3 mg). The serum contained 7.9% protein.)

Sheep serum added (ml)	0	0.2	0.4	0.8	1.6	0	0.8
Cetavlon added (mg)	—	—	—	—	—	1.6	1.6
CO ₂ (μl) evolved after 5 min	3	11	23	37	52	66	52
10 min	6	20	50	86	117	149	133
15 min	7	32	79	120	166	186	176

Procedure for the determination of glutamine and glutamic acid

Data presented in the previous section indicate that Gale's conditions (pH 4.5, 30°), though satisfactory for the determination of glutamic acid, are not optimal for the decomposition of glutamine. A less acid medium of pH 4.9 and a temperature of 40° is therefore proposed. A pH higher than 5.0 would be disadvantageous with regard to the manometric determination of CO₂, because at such a pH the medium would retain CO₂. Furthermore, addition of cetavlon is recommended if the material to be examined does not contain some protein. Extracts of animal tissues usually accelerate the decarboxylation of glutamine sufficiently to make the addition of cetavlon unnecessary.

Reagents

Bacterial suspension The stock suspension of *Cl. welchii* is diluted with 0.2 M acetate buffer (pH 4.9) to contain c. 25 mg dry bacteria/ml. If kept in a refrigerator the suspension may be used for about a month.

Stock acetate buffer 160 ml 3 M Na acetate are mixed with 100 ml 3 M acetic acid. This buffer (pH 4.9, 3 M) is diluted to a final concentration not exceeding 0.2 M, since higher concentrations inhibit the enzyme.

Cetyltrimethylammonium bromide Commercial cetavlon in water (2%) served as a stock solution. According to the makers, cetavlon contains about 70% of cetyltrimethylammonium bromide. A purer preparation (about 97%), kindly supplied by Mr S. Ellingworth of Imperial Chemical (Pharmaceuticals) Ltd, did not appreciably differ from commercial cetavlon in its action on the decarboxylation of glutamine.

Manometric arrangement Conical flasks provided with a side arm are used. The solution to be examined is placed in the main compartment. If very acid or very alkaline it is first adjusted to c. pH 4.9 by the addition of HCl or NaOH, and finally by 3 M acetate buffer, usually $\frac{1}{2}$ vol. The total volume of the fluid in the main compartment should not exceed 4 ml. The side arm contains 0.5 ml of bacterial suspension. Two types of control are required, one to measure the gas exchange of the bacteria alone, the other to measure the gas exchange of the solution to be examined. The first contains the bacterial suspension in the side arm and 4 ml 0.2 M acetate buffer in the main compartment. The second

contains a sample of the unknown solution, treated in the same way as in the test (no bacterial suspension in the side arm). The manometers are shaken at 40°, the contents of the side arm are mixed when equilibrium is established, and readings are taken until evolution of CO₂ ceases. Glutamic acid usually reacts completely within 15 min, glutamine within 20–30 min.

The control containing bacteria alone usually gave a small negative pressure, e.g. 4 μl in the first 30 min and 6 μl in the second 30 min, and it contained no more than a trace of NH₃ (4 μl). The gas exchange in the second control, also, is often negligible. An O₂ uptake, however, may occur when tissues are examined in which the enzymes have not been inactivated (see below).

Determination of NH₃ On completion of the CO₂ evolution the cups are detached from the manometers, and NH₃ is determined in the solutions by steam distillation *in vacuo* according to Parnas (1934). A modified apparatus which allows the collection of 20 ml of distillate was used.

Calculation For convenience the amounts of CO₂ and NH₃ are both expressed in μl. After correction for blanks, the NH₃ produced in the unknown solution is equivalent to the glutamine, and the CO₂ to glutamine + glutamic acid.

Recovery of glutamine

Recovery of glutamine from pure solution was satisfactory as shown in Table 7. The errors in eight determinations did not exceed 4% in the yield of CO₂ and 6% in the yield of NH₃.

Table 7 *Recovery of glutamine from pure solutions*

(Procedure as described in text)

Amount of glutamine analyzed (ml 0.01N solution)		2	1	0.5	0.25
Glutamine expected (μ l) (allowing for 93% purity)		416	208	104	52
CO ₂ (μ l) evolved	Duplicates	416	212	105	56
		419	212	103	50
Average		418	212	104	53
NH ₃ (μ l) found	Duplicates	420	210	106	54
		400	216	110	50
Average		410	213	108	52

Table 8 *Changes in the concentration of glutamic acid and glutamine in animal tissues on storage*

(Procedure as described on p 56 except that the tissue was not placed in liquid air 'fresh' tissue was placed in iced water within a few minutes of death of the animal, and analyzed after being kept in the ice for about 50 min. 'Stored at 25°' refers to a sample of the same liver kept in ice for the same time and then left in a covered vessel at 25° for 24 hr.)

		Substance (μ l/g wet liver)		
		Glutamic acid	Glutamine	Preformed NH ₃
Liver I	Fresh	131	35	59
	Stored at 25°	225	14	386
Liver II'	Fresh	146	24	87
	Stored at 25°	305	15	305
Liver III	Fresh	84	29	36
	Stored at 25°	152	5	278
Spleen	Fresh	137	15	34
	Stored at 25°	179	~0	492

Specificity

The following substances, in quantities of 5 mg each, did not produce NH₃ or CO₂ under the conditions of the glutamine determination

Amino acids glycine, DL alanine, DL-citrulline, L arginine, L phenylalanine, L tyrosine, L tryptophan, L cysteine, L cystine, DL methionine, L histidine, L aspartic acid

Acid amides L asparagine, urea, nicotinamide

Purine derivatives adenine, guanine, uric acid, allantoin, adenylic acid (muscle), adenylic acid (yeast), adenosinetriphosphate

Miscellaneous glucosamine, nicotinic acid, creatine, creatinine, succinimide

Preparation of animal tissues for the determination of glutamic acid and glutamine

Preliminary experiments indicated that special precautions have to be taken when glutamic acid and glutamine are to be determined in animal tissues. The concentration of these two substances can undergo rapid changes, owing to the presence of proteolytic enzymes and glutaminase in these materials. Inactivation of the enzymes is not always practicable because operations commonly employed for this purpose are liable to destroy glutamine or to generate glutamic acid from protein or peptides.

When intact fresh tissue was kept at room temperature the glutamic acid increased and the glutamine fell (Table 8). In minced tissue, mixed with 2 parts of 0.1N HCl (resulting pH 3.7), both glutamic acid and glutamine rapidly increased even if the suspension was kept in the refrigerator (Table 9). The parallel increase in amino N indicates that the increase is due to proteolysis.

Table 9 *Changes in the concentration of glutamic acid and glutamine in acidified liver suspensions*

(Chilled sheep liver ground with 2 parts 0.1N HCl (pH of suspension 3.7) electrometrically) Amino N determined with nitrous acid method after deproteinization with trichloroacetic acid)

Treatment of liver suspension	Substance (μ l/g wet wt)			
	Glutamic acid	Glutamine	Preformed NH ₃	Amino N
Fresh suspension	110	29	69	920
Stored 24 hr near 0°	216	57	195	5,040
Stored 24 hr at 25°	442	148	250	13,900

When 0.1N HCl was replaced by 0.3N HCl, the pH of the resulting tissue suspension being 2.2, an increase, though smaller, of both glutamic acid and glutamine was observed on 24 hr storage in the refrigerator. On the other hand, the concentrations of glutamic acid and glutamine in a suspension containing 1 part of tissue and 2 parts of 0.5N HCl (pH about 1.0) remained almost constant in the refrigerator for 24 hr. After 48 hr, however, some of the glutamine had been converted into ammonium glutamate. The higher amount of

acid (0.5N) also inhibited the rise in 'preformed' NH_3 , i.e. the NH_3 present in the tissue before the addition of *Cl welchii* (Table 10)

Table 10 *Changes in the concentration of glutamic acid and glutamine in acidified liver suspensions*

(Rabbit liver frozen in liquid air for 20 min, thawed, minced and mixed with 2 vol 0.5N HCl)

	Substance (μg /g wet wt)		
	Sum of glutamic acid and glutamine	Glutamine	Preformed NH_3
Examined immediately	138	27	50
Stored 24 hr near 0°	138	28	48
Stored 48 hr near 0°	139	12	61
Stored 24 hr at 20°	152	—	—

A further error to be avoided is a loss of glutamic acid and glutamine, which occurs when tissue is packed in ice. The water from the melting ice penetrates into the tissue and causes it to swell. At the same time, amino acids (and other substances) diffuse into the hypotonic medium. The result is that the concentrations of the amino acids/unit weight of tissue fall. The magnitude of the changes is illustrated by the following example: 69 g of liver, placed in 200 ml water and 200 g ice in a Dewar vessel, weighed 99 g after 24 hr, whilst 52 mg of glutamic acid had diffused into the water.

A complication interfering with the manometric CO_2 determination is the O_2 absorption which extracts or suspensions of liver, and to a less extent of testis, exhibit when shaken in air at pH 4–5. Most tissues tested (kidney, cortex and medulla, brain, spleen, heart, skeletal muscle, pancreas, thyroid gland) have no appreciable O_2 uptake under the experimental conditions. Sheep liver absorbed about 0.7–1.25 $\mu\text{L}/\text{mg}$ dry wt/hr at 40° , without yielding CO_2 . Often the rate increased with time. The substrate of this oxidation has not been identified. In some experiments the rate of the O_2 uptake increased on addition of the bacteria, and the tissue suspension alone is therefore not a satisfactory control to measure the O_2 uptake. The difficulties are overcome by filling the gas space of the manometers with N_2 .

Procedure for the preparation of animal tissues On the basis of the above observations the following procedure was adopted. The material, cut into 5–10 g pieces, was thrown into liquid air as quickly as possible after death, and left there for at least 15 min. After removal from the Dewar vessel it was minced in a cooled tissue grinder as soon as the consistency permitted, transferred to a tared beaker, weighed, and 2 ml of cooled 0.5N HCl/g tissue were added with thorough mixing. If the suspension could not be analyzed immediately it was stored in a refrigerator. For the determination of glutamic acid and glutamine, 2 ml of suspension were pipetted into the main compartment of a conical Warburg vessel, followed by 0.1 ml of 0.1% aqueous methyl orange and just sufficient N NaOH to change the indicator to yellow (usually about 0.6 ml). Next, 0.2 ml 3M acetate buffer (pH 4.9) was added to the main compartment. The further procedure was as already described, except that the gas space of the vessels was filled with N_2 , and the centre contained a stick of yellow phosphorus.

Recovery of glutamine added to tissue suspensions When known amounts of glutamine were added to acidified liver suspensions recovery was satisfactory, for example, 73 μL added, 74 μL recovered, 36.5 μL added, 41 μL recovered.

Presence of inhibitors Like other analytical methods based on the use of enzymes, the present procedure cannot be applied to solutions containing inhibitors of the enzymes concerned. If the presence of inhibitors in the unknown material cannot be excluded, glutamine should be added and its recovery tested. As the decarboxylation of glutamine is more sensitive towards some inhibitors than that of glutamic acid, the latter is not always a suitable test substrate. In pure solutions various indicators (0.01%), such as phenolphthalein, bromocresol green, bromocresol purple and anionic detergents such as sodium dodecylsulphate (0.05%), were found to inhibit the decarboxylation of glutamine. These inhibitions were largely abolished by the addition of cetavlon (0.05%) or serum (1 ml to 3–4 ml solutions), and the addition of one of these reagents may therefore be advisable if inhibitors are present. If serum is used, a blank determination is required, human serum contains about 8 mg of glutamine and 2 mg of glutamic acid/100 ml.

DISCUSSION

The main advantage of the present method over some previous techniques is the higher degree of specificity, coupled with relative simplicity. Vickery, Pucher, Clark, Chubnall & Westall (1935) deprecated the general application of their procedure (heating for 2 hr at 100° at pH 6.5 and estimating the increase in NH_3N) to unknown material because of lack of specificity. They reported that urea and allantoin yielded NH_3 . Bartley & Krebs in unpublished experiments found that nicotinamide methochloride formed about 0.25 mol of NH_3 when subjected to the procedure of Vickery *et al*. As nicotinamide methochloride is analogous to the pyridine nucleotides it is possible that these coenzymes also form NH_3 . The specificity of Archibald's glutaminase from kidney is likewise imperfect, in that it is contaminated with deaminases liberating NH_3 from amino purine nucleotides.

It cannot be claimed that the specificity of the glutaminase of *Cl welchii* (strain S R 12) is absolute. However, it seems to be very high, since so far no substance apart from glutamine has been found to form NH_3 under the experimental conditions. Moreover, in a large number of analyses of animal and plant tissues, no material was encountered where the yield of NH_3 exceeded that of CO_2 . If substances other than glutamine can produce NH_3 with the bacterial enzyme, an excess of NH_3 over CO_2 might have been expected.

SUMMARY

1 L Glutamine yields one molecule each of NH_3 and of CO_2 when added to washed suspension of *Clostridium welchii* in acid solution. The formation of NH_3 precedes that of CO_2 . The 'glutaminase' of the organism is highly specific, none of many substances tested was found to yield NH_3 .

2 The method for the determination of glutamine and glutamic acid combines the principles used by Gale and by Archibald. The sum of the substances is determined according to Gale by manometric determination of the CO_2 evolved on decarboxylation. The NH_3 is then estimated in the solution treated with *Cl. welchii* and in a sample not treated with the bacteria. The difference represents the glutamine.

3 The conditions affecting the quantitative reaction of glutamine were examined. In pure solutions

glutamine reacted much more slowly than glutamic acid, and the rate of reaction of glutamine, and to a smaller extent that of glutamic acid, was much (in some cases more than tenfold) accelerated by cetyltrimethylammonium bromide, serum or tissue extracts. In the presence of these substances the maximum rates of the decarboxylation of glutamine and glutamic acid were approximately equal.

4 The slow reaction of glutamine seems to be due, under some conditions, not to the low activity of the glutaminase or glutamic decarboxylase, but to permeability barriers. Removal of these barriers would account for the accelerating effect of cetyltrimethylammonium bromide.

5 The glutamine and glutamic acid content of animal tissues rapidly increases after death owing to autolysis. A procedure has been elaborated which minimizes the effects of autolytic enzymes.

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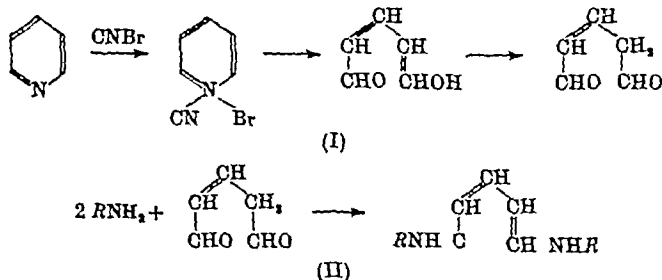
The Recovery of l-Nicotine from Animal Tissues and its Colorimetric Micro-estimation

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Some heterocyclic substances containing a trivalent nitrogen atom react with 1,2,4-chlorodinitrobenzene (Vongerichten, 1899; Reitzenstein, 1903), cyanogen bromide, phosphorus pentachloride (König, 1904)

action of cyanogen bromide on furfural, when the ring opens at the cyclic oxygen atom. The glutamic derivative obtained in this way will condense with certain aromatic amines to form



and other substances to produce an unstable pentavalent nitrogen derivative which immediately changes to an open chain derivative of glutamic aldehyde (see (I)). A similar change occurs in the

stable coloured compounds (see (II)). The tint and intensity of colour produced depend primarily upon the structure of the heterocyclic base and the amine. In the cases which have been investigated

the development of colour is also influenced by the pH and the concentration of inorganic ions in the solution

This reaction has been widely used for the detection and estimation of pyridine derivatives of biological importance. The procedures adopted have been reviewed by Bacharach (1941) and Waisman & Elvehjem (1941). Barta & Marschek (1937) estimated nicotine in tobacco by measuring the colour produced by the action of cyanogen bromide and β -naphthylamine on extracts of the plant. This procedure has now been developed for the accurate estimation of nicotine recovered from animal tissues by a distillation procedure. Quantities up to 800 μ g of the base were recovered from 2 to 10 g wet weight of tissues in 15 ml of distillate. Quantities of 2 μ g/ml distillate and upwards were estimated with an error of $\pm 1\%$.

EXPERIMENTAL

Reagents (1) *l*-Nicotine, purified by distillation under reduced pressure, b.p. 122–123°/16 mm, n_D^{20} 1.5230. Standard solutions were prepared by dissolving a weighed quantity of freshly distilled nicotine in water immediately before use.

(2) Cyanogen bromide, prepared in aqueous solution by the method of Larson & Haag (1944). This was only necessary for the estimation of small quantities of nicotine with maximum accuracy, and for most purposes the solution obtained by just decolorizing saturated bromine water with 10% (w/v) KCN solution was found to be suitable. This preparation was diluted five times with distilled water and adjusted to pH 6 with KCN solution. If kept at 0° both solutions lasted for a week without deterioration.

(3) Ethanolic β -naphthylamine. β -Naphthylamine was recrystallized from ethanol immediately before use to remove the coloured substances which are rapidly formed from it on exposure to light. The purified amine was made up in 0.2% (w/v) solution in 95% ethanol before use.

(4) 0.2M Sorensen's phosphate buffer, pH 5.9–6.1

(5) Acid aqueous ethanol. This consisted of a mixture containing 9 parts by volume 50% ethanol and 1 part of a mixture of equal volumes of glacial acetic acid, concentrated HCl and amyl alcohol.

The reagents were used in the following order and proportions: nicotine solution, 1.0 ml; buffer, 0.2 ml; CNBr solution, 0.27 ml.; ethanolic β -naphthylamine, 1.0 ml.

Factors influencing the rate of colour development and intensity

A number of workers (see Bacharach, 1941, Waisman & Elvehjem, 1941) have used this reaction for the estimation of nicotinic acid and nicotinamide. The more recent investigations have shown that the intensity of colour developed is markedly influenced by the pH in the solution and by the presence of inorganic ions. This was also found to be the case with *l*-nicotine. The addition of the phosphate buffer indicated above led to the development

of the maximal intensity of colour. This buffer was the sole source of inorganic ions present in the first stage of the estimation.

The rate of colour development, which is slow at room temperature, was increased by heating to 37°. At 37° and the optimum pH 5.9–6.1 it was found that the colour developed was yellow which gradually increased in intensity and changed to orange red. Measurements with a spectrophotometer showed that there is a peak of extinction between the wave lengths of 490 and 550 m μ . In view of this, green colour filters were used in subsequent investigations. Measurements were made with the Spekker photoelectric absorptiometer, using the green filter no. 5, and in routine visual colorimetry with the Duboscq type colorimeter. Chance's glass filter 0 Gr. 1 was employed. With the aid of the absorptiometer it was found that the intensity of colour reached a maximum in 2.5 hr. and remained constant up to 20 hr. A typical set of results is shown in Fig. 1. A series of tubes containing 300 μ g

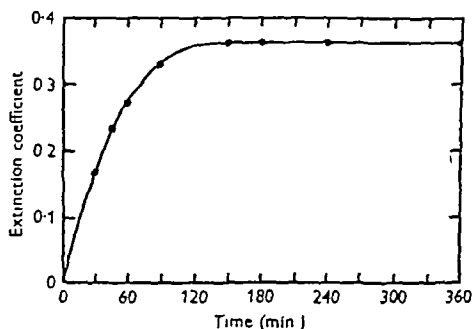


Fig. 1 Nicotine (300 μ g), cyanogen bromide and ethanolic β -naphthylamine. Relation between extinction coefficient and the time of heating at 37°.

of nicotine in 1 ml of solution was treated with appropriate volumes of reagents and kept at 37°. At intervals tubes were removed in duplicate, the contents made up to 20 ml with 50% ethanol and the optical density read in the absorptiometer. The relation between the extinction coefficient and the time of heating is shown.

If one of the above solutions is taken after a minimum of 2.5 hr. heating, made up to 20 ml with 50% ethanol and allowed to stand in the air, the second colour change takes place. A slow increase of optical density occurs and the tint changes to wards magenta. This change is greatly accelerated by the addition of acid aqueous ethanol (reagent 5) in the place of 50% ethanol. Fig. 2 shows the relation between the extinction coefficient and the time of keeping at room temperature of solutions from the above series which had been heated for 2.5 hr. at 37° and then made up to 20 ml with acid aqueous ethanol. The diagram shows that the change is complete within 2 hr. However, in practice it is

not necessary to wait for 2 hr before reading, for the relative rate of increase of extinction is independent of the original concentration of nicotine. Provided, therefore, that the acidified ethanol is added to all the solutions at the same time, they may be compared with a standard, prepared simultaneously with them, at any time after the addition. Absolute readings with the absorptiometer can, of course, only be taken after the full 2 hr has elapsed.

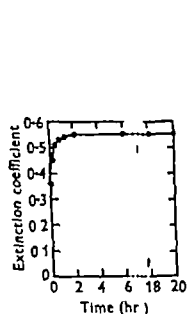


Fig 2

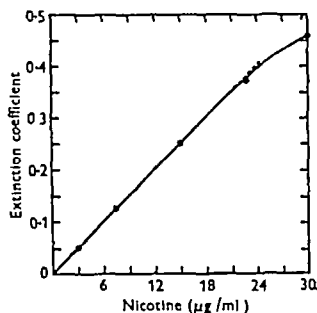


Fig 3

Fig 2 Nicotine (300 µg), cyanogen bromide and ethanolic β naphthylamine. Relation between extinction coefficient and the time of keeping with acid ethanol at room temperature after the full development of the primary colour at 37° and pH 6

Fig 3 Relation between the extinction coefficient of the reaction mixture and the concentration of nicotine

Beer's law As is shown by Fig 3, there is a linear relationship between colour and nicotine concentration over a wide range (in this case 1–20 µg/ml)

The estimation of micro quantities of nicotine

It is clear that, if it is contained in a suitably small volume, about 1 µg of nicotine may be estimated by this method, using either a direct vision colorimeter with small cups, capacity c 2 ml, or a photoelectric colorimeter with 2 ml cuvettes, with suitable colour filters. The error is less than 5%.

As will be seen in a later section, the minimal volume in which all the nicotine may be recovered from animal tissues is 15 ml, so that when very small amounts of nicotine are present it may be necessary to concentrate the solution. This may be done by reducing the pH to 4 with HCl and evaporating under reduced pressure.

The recovery of nicotine from tissues

Nicotine is recovered from tissues by taking advantage of its volatility in steam. The nature of the tissues under investigation and the use to be made of the distillate will determine the precise procedure employed. For the purpose of colorimetry

it is desirable to have the nicotine in a minimum volume of distillate. It was found that the base could be recovered quantitatively from the tissues of the nematode *Ascaris lumbricoides* var *suis* by a modification of the procedure of Werle & Becker (1942). The distillation apparatus, which is based on the Pregl micro Kjeldahl apparatus, is illustrated in Fig 4. Fresh worm tissue (2–10 g), containing nicotine, was chopped into the detachable flask A. The following reagents were then added: (1) distilled water to a total weight of 25 g, (2) 11 g of NaCl, and (3) 0.3 g of MgO.

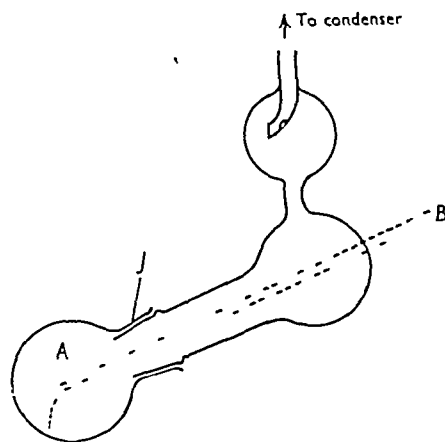


Fig 4 Diagram of distillation apparatus for the recovery of nicotine from tissues

Table 1 Recovery of nicotine from tissues of *Ascaris*

l Nicotine added (µg)	Wet wt tissues (g)	Recovery of nicotine (%)
30	5.7	100
30	6.4	100
60	6.7	99
60	5.1	100
150	6.0	100
300	5.0	98
300	5.2	100
600	5.5	97
600	5.5	99

The flask was attached to the apparatus by the ground glass joint J and supported by a ring and gauze. The mixture was then heated by a small gas flame, and 15 ml. of distillate were collected, care being taken to avoid frothing beyond the first bulb of the still head. Some examples of the recovery of nicotine are given in Table 1. In this experiment small volumes of nicotine solution, c 1 ml, were added to chopped *Ascaris* tissues, incubated at 37° for 6 hr, and then distilled. The final volume of solution for estimation was 50 ml.

The process of distillation was slow, taking about 30 min for the complete operation. Recoveries adequate for exploratory and comparative work may be obtained by a much more rapid steam distillation procedure in which the flow of vapour is supplemented by the passage of steam through the tube B (broken line, Fig 4). By this procedure about 75 % of the nicotine may be recovered in 5 min in 15 ml of distillate.

SUMMARY

A method is described for the recovery of nicotine from tissues and its estimation in quantities from 1 μ g.

The author is grateful to Prof A C Chubb, F R S, for his interest in this work, which was part of a programme of investigations into the mode of action of anthelmintics, carried out for the Agricultural Research Council.

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Decomposition and Synthesis of Cozymase by Bacteria

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Investigations concerning the metabolism of nicotinic acid and its derivatives have been surveyed recently by Schlenk (1945), and, in the case of micro organisms, by McIlwain (1947). In the latter account, attention was directed to the synthesis, inactivation and interconversion of coenzymes I and II by micro organisms, and particular efforts were made to obtain values for the rates at which these changes occur. Although many papers bearing on this subject were assessed, few if any were found to contain all the data required for calculating unequivocally the rates at which these important processes take place. On the other hand, such estimates of the rates as could be made by supplying likely values for missing data suggested the existence of a group of reactions with velocities of a few μ mol/mg dry wt of organism/hr. The possible significance in bacteria of reactions of this magnitude has been discussed elsewhere (McIlwain, 1946b).

Studies have now been made of the behaviour of bacteria towards nicotinic acid derivatives, especially cozymase. This behaviour has been found to be dominated in many organisms by reactions which are potentially much more rapid than those inferred previously. Before studying the slower reactions (for which, also, evidence has been obtained) the course and products of the more rapid reactions have been examined, and are reported in this paper.

EXPERIMENTAL

Organisms. *A β* haemolytic streptococcus (R, the Richards strain, National Collection of Type Cultures no. 5631), two strains of *Streptococcus faecalis* (F 4208, N C T C no. 4208, FL, a laboratory strain), a *Staphylococcus aureus* (laboratory strain) and *Proteus morganii* (N C T C no. 2818) were grown in the casein yeast medium of McIlwain (1946a), modified as follows: in place of NaOH, 0.5 M NaHCO₃ (additional 6.8 ml/100 ml.) was added, the initial pantothenate content was 10⁻⁶ M, the quantity of yeast preparation was 2.5 ml/100 ml., and of the group A addenda only riboflavin, aineurin, KH₂PO₄, tryptophan, methionine, MgSO₄, Fe(NH₄)(SO₄)₂ 6H₂O and cystine were included. *Escherichia coli* (N C T C no. 4074) was grown in the mixture of inorganic salts and glucose of Dorfmann & Koser (1942).

Lactobacillus arabinosus 17-5 was grown in a medium elaborated for the use of the organism in assaying nicotinic acid (see below), and containing the quantities of nicotinic acid specified in the individual experiments described. Its pH fell from 6.8 to 5 during growth. *Haemophilus para influenzae* was also grown in the corresponding assay medium, with defined quantities of cozymase as V factor.

Reaction with cozymase. Bacteria were normally harvested by centrifuging, washed twice with 0.9 % NaCl, suspended in that solution and a small sample taken for dry weight estimation. The suspensions were distributed between experimental vessels, which, for anaerobic experiments, were Warburg flasks with yellow P in a centre well. Saline was that of Krebs & Henseleit (1932).

Microbiological assays. Nicotinic acid was determined using *Lactobacillus arabinosus* 17-5 in very nearly the way

described by Barton Wright (1946) (see McIlwain & Stanley, 1948). V factor was estimated by growth of *Haemophilus parainfluenzae* (NCTC no 4101) in a medium similar to that of Hoagland & Ward (1942), growth, however, was measured photoelectrically. Cozymase in concentrations of $0.3\text{--}2 \times 10^{-2} \text{ M}$ was used as source of V factor in obtaining reference curves, and results are expressed in terms of cozymase. The inoculum was 0.05 ml of a culture grown for 24 hr in the assay medium with 10^{-2} M cozymase. Inoculated cultures of 10 ml final volume were incubated in 50-ml conical flasks at 37° for 30–48 hr.

Determination of cozymase using apozymase. Though the determination of cozymase using apozymase (Euler, 1936, Myrback, 1933) requires care, the method appears much more reliable than was suggested by Jandorf, Klemperer & Hastings (1941). By adopting precautions specified by Axelrod & Elvehjem (1939) and others noted below, we have consistently obtained reliable results. Our experience and practice have been in some cases similar to, and, in others, different from that of Schlenk & Schlenk (1947), whose paper appeared while the present one was in preparation.

Preparation of apozymase. Fresh brewer's yeast was dried in air and then over CaCl_2 , it can be kept in this form for some months. The washing, drying, rewashing and redrying (all these stages were found necessary) to yield apozymase were carried out according to Axelrod & Elvehjem (1939) and in one day. The product was kept in a stoppered bottle over CaCl_2 in a refrigerator for periods up to a fortnight.

It was found necessary to carry out the preparation of apozymase at room temperature ($15\text{--}20^\circ$). When, in trying to obtain a more stable preparation, washing was performed with ice water, the apozymase obtained was deficient in cozymase, but its response to certain solutions containing cozymase increased with time. Such behaviour has been observed by Euler & Adler (1938), Euler, Adler & Eriksen (1937) and by Lennerstrand (1941), and interpreted as due to synthesis of cozymase. We have not found such behaviour in apozymase prepared as described in the preceding paragraph, and have therefore not found it necessary to adopt a method such as that of Lennerstrand's (1941) 'pyocyanin system', which is much less sensitive to cozymase than is the normal apozymase system.

Preparation of hexosediphosphate. Neuberg's (1942) method was followed, using fresh baker's yeast, fermentation was complete in 5 hr and the yield of calcium hexosediphosphate from 200 g of sucrose was c. 16–20 g. A solution of sodium

hexosediphosphate was prepared freshly each day, the Ca salt (208 mg) was dissolved in 0.49 ml of ice cold N HCl and the theoretical quantity of sodium oxalate, in 3.5 ml water, added gradually. The pH was brought to 7 by N NaOH (0.4 ml), the mixture left cold for 10 min and centrifuged. The solution was taken to pH 6.2 with N HCl (c. 0.15 ml) for use in the apozymase system, it contained c. 4 mg organic P/ml.

Cozymase determination. This followed Axelrod & Elvehjem's (1939) description, but was performed in Warburg vessels with one side arm and of total volume c. 18 ml. With the apozymase used, response of the system at 30° to cozymase was found to be maximal with phosphate concentrations between 0.055 and 0.07 M, and the quantity of phosphate buffer added for cozymase determination was such as to give a final concentration of 0.065 M. This concentration is higher than that adopted by Axelrod & Elvehjem (1939), but other reagents were used in concentrations described as typical by those authors. If the solutions whose cozymase content was being determined were significantly buffered, they were brought to pH 6.2, and if their phosphate content was more than 0.02 M, that of the remainder of the system was reduced accordingly. With 0.2 g of apozymase/vessel, the system was used for determination of up to 15 or 20 μmol of cozymase, and standard solutions of 7.5 and 15 μmol of cozymase were included with each batch of unknown solutions. It is necessary to avoid citrate buffers in solutions whose cozymase is being determined by apozymase.

Other determinations of cozymase were made by dismutation of hexosediphosphate (method and muscle preparation of Jandorf *et al.* 1941) and reaction with hypsulphite, following Warburg & Christian's (1936) description.

Cozymase specimens. Pure cozymase has not been obtained, and unless otherwise stated, specimen A of Table 1 has been used, but its behaviour has been compared with two independent specimens B and C of Table 1. The different methods of estimating the cozymase content of specimen A, quoted in Table 1, gave values differing by not more than 6%, and their mean value of 40% purity has been adopted in interpreting results with this specimen. The principles underlying methods I and II are entirely different from each other and from that underlying methods III and IV. The approximate agreement between methods I and II suggests that specimen A contains practically all its nicotinic acid as a quaternary salt such as cozymase. The approximate

Table 1. Analytical data concerning cozymase specimens

Determination and standard value		Cozymase specimen		
		A	B	C
I	Acid formation in reaction with $\text{Na}_2\text{S}_2\text{O}_4$ ($\mu\text{mol}/\text{mg}$)	1.75	0.50	1.80
	Purity by comparison with theoretical value of 4.5 $\mu\text{mol}/\text{mg}$ ($\text{C}_{12}\text{H}_{17}\text{O}_{14}\text{N}_7\text{P}_2$, mol wt 663) (%)	37	11	40
II	Nicotinic acid content (%)	8.0	—	—
	Purity (theoretical value 18.6%) (%)	43	—	—
III	Catalytic activity in apozymase system ($\mu\text{mol CO}/\mu\text{g/hr}$) (s.d. of 15 results with specimen A, 0.18)	1.47	0.44	1.75
	Purity (by comparison with value of 4.0 derived from Axelrod & Elvehjem, 1939) (%)	37	11	44
IV	Catalytic activity in Jandorf <i>et al.</i> (1941) system ($\mu\text{mol CO}/\mu\text{g/hr}$)	0.89	—	—
	Purity (by comparison with value of 2.15, derived from Jandorf <i>et al.</i> 1941) (%)	41	—	—

agreement between methods II, III and IV implies that the specimen contains relatively little material containing nicotinic acid which is not cozymase. The agreement between method III and the other methods justifies our dependence on method III in most of the subsequent work. We have also shown (Table 7) that the nicotinic acid grouping of specimen A exists as its amide, without detectable free acid (cf Schlenk, 1945).

Extraction of bacterial cozymase. Extractions were performed aerobically in phosphate buffer, pH 6.2, as this is the medium in which cozymase is estimated in the apozymase system, and pH 6.2 is close to that of optimal stability of cozymase. Previous investigators have heated for short periods at temperatures of 70–100° in order to obtain cozymase from natural materials, and we have examined the yield of cozymase from streptococci under such conditions. Results quoted in Table 2 show that heat treatment for 8–30 min at these temperatures yields extracts containing about the same quantity of cozymase, but that 15 min at 70° is optimal. The quantity of cozymase so obtained was found to be nearly the same as that extracted from the cells by grinding with glass. Heating at 70° for 15 min has therefore been employed throughout the studies reported in this paper for extracting cozymase. Table 2 shows that 5% of the substance may be lost under these conditions, but values quoted have not been corrected for such loss.

RESULTS

Inactivation of cozymase

Behaviour of various bacterial species. Washed suspensions of bacteria were incubated in buffered salt solutions containing 10^{-5} – 10^{-4} M cozymase. The majority of species inactivated cozymase (Table 3). Reaction at the rate of 5–30 $\mu\text{mol}/\text{mg}/\text{hr}$ was found in β haemolytic streptococci, *Haemophilus parainfluenzae*, *Staphylococcus aureus* and *Proteus morganii*. *Escherichia coli* and *Streptococcus faecalis* were less active, *Lactobacillus arabinosus* 17–5 was the only organism of those examined with activity $<0.5 \mu\text{mol}/\text{mg}/\text{hr}$.

Circumstances affecting the reaction. Most of the following investigations were with streptococci. The reaction reached its greatest velocity at a pH near 6 or 7 (Table 4, see also Table 8). We have usually studied it at pH 7–7.5 in order to assess its relation to growth. With high concentrations of cozymase, very rapid rates of inactivation were reached by all three species examined. In Table 5, cozymase at an initial concentration of 1.2×10^{-3} M is seen to be

Table 2 *Extraction and stability of cozymase from streptococci*

(Streptococci throughout were the Richards strain, grown in the casein yeast medium for (in different batches) 20–40 hr.)

Material extracted or treated	Conditions of extraction or treatment	Cozymase		
Streptococci, batch A	At 70°, 15 min, 2.4 mg cells/ml	6.3		
	At 70°, 30 min, 2.4 mg cells/ml.	5.9		
	At 85°, 15 min, 2.4 mg cells/ml.	5.8		
Streptococci, batches B and C	At 70°, 15 min At 85°, 15 min At 100°, 8 min	(Batch B) (Batch C)		} $\mu\text{mol}/\text{mg cells}$
		9.8	4.9	
		9.0	4.3	
		8.5	3.8	
Streptococci, batch B	Ground with glass	9.5		}
	Ground with glass and extract heated at 70°, 15 min	9.2		
Streptococcal extract	None	(a)		}
	Heated at 70°, 15 min	94% of (a)		
Cozymase	None	(b)		}
	Heated at 70°, 15 min	94–97% of (b)		
Streptococci with added cozymase				
Cells (4.8 mg)	At 70°, 15 min	48		} μmol .
Cozymase	At 70°, 15 min	82		
Cozymase with cells (4.8 mg)	At 70°, 15 min	126		

The rapid inactivation of cozymase by streptococci makes it possible that losses may occur by enzymic activity during the heating to 70°, but we have not found this to be the case. The extraction has been carried out in small volumes of liquid (usually <2 ml) placed in tubes in a water bath at 70°. Temperatures approaching 70° are quickly attained in the tube, and cozymase added to the system is recovered satisfactorily (Table 2).

inactivated at over 440 $\mu\text{mol}/\text{mg}/\text{hr}$. The system does not approach saturation even at that concentration, values for $(\log a/b)/t$ (a = initial, b = concentration at time t) are not falling at the higher concentrations of Table 5A. In Table 5B, also, the velocity is proportional to the initial concentration of cozymase, except with the lower concentration

Table 3 *Bacterial inactivation of cozymase*

(Reactions were at 37°, with shaking, in tubes or Warburg vessels. The latter, with yellow P in a centre well and N₂ in the gas space, were employed for anaerobic reactions. Changes in cozymase refer to the whole reaction mixture including the cozymase of the cells.)

Organism and dry wt of cells/ml	Reaction mixture		Reaction performed aerobically (A) or anaerobically (AN)	Rate of change in cozymase ($\mu\text{mol}/\text{mg}/\text{hr}$)
	Cozymase ($\text{M} \times 10^{-5}$)	Other constituents		
<i>β</i> Haemolytic streptococcus (R), 3.9 mg/ml	2.4	0.02M Na and K phosphates, pH 7.6	A	-29
<i>Streptococcus faecalis</i> (FL), 3 mg/ml	1.2	0.03M Na and K phosphates, pH 7.6, with saline	AN	-1.8
	1.2	Do -	A	-1.7
<i>Staphylococcus aureus</i> (E), 3.2 mg/ml	1.2	Do -	AN	-7.0
	1.2	Do	A	-5.2
<i>Proteus morganii</i> (2818), 1.3 mg/ml	1.2	Do	A	-4.2
	1.2	Do	AN	-4.5
<i>Lactobacillus arabinosus</i> 17-5, 21.5 and 45 hr old, 2.3 mg/ml	6	As above, also with glucose (0.05M)	A	<0.5
<i>Lactobacillus arabinosus</i> 17-5, 40 and 44 hr old, 3.6 mg/ml	6	As above, pH 5 and 7	A	<0.5
<i>Haemophilus parainfluenzae</i> , NCTC 4101, c 2 mg/ml	6	0.03M Na and K phosphates, with saline, pH 6 and 7	A	-0.7 to 8.4
<i>Escherichia coli</i> , 7 mg/ml	2.4	As above at pH 7.5	A	-0.6
	2.4	Do	AN	-3.5
	2.4	As above with glucose (0.02M)	AN	-1.1

Table 4 *Cozymase inactivation by streptococci: different specimens, pH, concentrations and temperatures*

(Reactions were aerobic and in phosphate saline, except * which was in acetate buffer. Cozymase specimens: see Table 1.)

Specimen	Cozymase		Organism (batch)	Temp	pH	Rate of inactivation ($\mu\text{mol}/\text{mg}/\text{hr}$)
	Initial concentration (M)					
A	4×10^{-4}		R (a)	10	7.5	172
A	4×10^{-4}		R (a)	20	7.5	267
A	4×10^{-4}		R (a)	37	7.5	390
A	4×10^{-4}		R (b)	37	7.5	295
C	3.6×10^{-4}		R (c)	37	7.5	160
C	3.6×10^{-4}		R (d)	37	7.5	266
B	3.6×10^{-4}		R (d)	37	7.5	250
A	1.2×10^{-3}		FL	37	7.5	288
A	3.6×10^{-4}		F 4208	37	7.5	144
A	4×10^{-4}		R (e)	37	5*	37
A	4×10^{-4}		R (e)	37	6	183
A	4×10^{-4}		R (e)	37	7	159
A	4×10^{-4}		R (e)	37	8	84

when measurements were less accurate. Different cozymase specimens and different streptococcal strains behaved similarly, and the reaction was still rapid at 10° (Table 4).

The course of change in cozymase in one reaction mixture was followed both by apozymase and by the muscle preparation of Jandorf *et al.* (1941). Results obtained by the two methods did not differ by more than 6%.

Effects of other substances on the reaction. Inactivation of cozymase in the presence and absence of oxygen is compared with several organisms in Tables 3 and 6. Differences between the two conditions were in general not striking and often within experimental error. Any difference was in the sense of greater loss anaerobically, the greatest contrast was found with a *Streptococcus faecalis* (Table 6) and *Escherichia coli* (Table 3).

Table 5 *Course of the streptococcal inactivation of cozymase*

(In Exp A, organisms of strain R were grown in 500 ml of standard medium, collected, washed twice in 0.5% NaCl and the bulk, of dry wt 190 mg, suspended in 10 ml of 0.5% NaCl and mixed with cozymase (33 μ mol) in 10 ml of 0.04M Na and K phosphates, pH 7.6. The mixture was shaken at 37° in air, and specimens (0.2 ml) were taken at the intervals indicated, diluted in cold water, and their cozymase determined in the apozymase system.

For Exp B, a smaller quantity of a similar reaction mixture was serially diluted to give the four different concentrations of cozymase (and of organisms) which are indicated.)

Reaction period (min)	Cozymase content		Mean $Q_{\text{Coz.}}$ (m μ mol./mg/hr)	$\frac{\text{Log } a/b}{\text{Time (hr)}}$
	$\mu\text{mol in whole}$	$M \times 10^{-4} \text{ (c)}$		
	Exp A			
0	33.3 (a)	16.7	—	—
20	13.5 (b_1)	6.75	-155	1.18
40	6.04 (b_2)	3.02	-117	1.11
60	2.28 (b_3)	1.24	-59.4	1.16
90	1.22 (b_4)	0.61	-16.7	0.96
Exp B				
15	—	12	-444	$Q_{\text{Coz.}}/c$ -37
15	—	4	-140	-35
30	—	1.33	-48	-36
60	—	0.44	-11	-25

Table 6 *Distribution of cozymase between bacterial cells and suspending fluid during its inactivation*

(Reactions were carried out in 2.5 ml of phosphate saline, pH 7.5.)

Reaction mixture			Reaction		Cozymase (μ mol)			
Organisms (mg dry wt)	Other reagents	Added cozymase (m μ mol)	Aerobic (A) or anaerobic (AN)	Time (hr)	In cells	In solution	Total	Loss
<i>Streptococcus faecalis</i>								
8.4	—	—	A	2	43	4	47	—
8.4	—	62	A	2	60	35	95	14
8.4	—	62	AN	2	49	33	82	27
8.4	—	62	AN	0.5	54	48	102	7
8.4	Glucose, 0.01M	62	AN	0.5	61	38	99	10
<i>Staphylococcus aureus</i>								
9.5	—	—	AN	2	53	< 1	53	—
9.5	—	30	AN	0.5	54	< 1	54	20
9.5	Glucose, 0.01M	30	AN	0.5	48	< 1	48	35
9.5	—	30	A	2	53	7	60	23
<i>Proteus morganii</i>								
3.7	—	—	AN	2	41	3	44	—
3.7	—	34	AN	2	37	3	40	38
3.7	—	34	A	2	38	2	40	38
<i>Escherichia coli</i> 4074								
7.4	—	—	—	—	26	—	26	—
7.4	—	93	AN	0.5	42	13	55	64
7.4	—	93	AN	2.0	40	< 1	40	79
7.4	Glucose, 0.01M	93	AN	0.5	58	14	72	47
7.4	—	93	A	2.0	56	1	57	62

Glucose did not have a large effect on the reaction, instances of greater loss in its presence are shown in Table 6 with streptococci and staphylococci. During these experiments both organisms caused a rapid breakdown of the added glucose, largely to lactic acid. Glucose, on the other hand, decreased the inactivation of cozymase brought about by *Esch*

coli. Sodium pyruvate, α ketoglutarate, and glutamine (all at 7×10^{-3} M) were not found to alter the rate of change in cozymase added anaerobically to suspensions of the R streptococci. Variation in the phosphate concentration of the medium between 0.008 and 0.08M caused no change in the reaction with cozymase.

Distribution of cozymase between cells and solution
The experiments of Tables 3-5 concerned reaction mixtures in which the added cozymase was in large excess over that of the bacteria, or in which the cozymase of the complete reaction mixture had been determined after liberating the substance from the cells. The experiments of Table 6 were performed with relatively little added cozymase, and the distribution of cozymase between the bacteria and the solution was examined. Added cozymase was found first to enter the cells and then to be lost without reappearing in solution. Much added cozymase could be lost with relatively little change in the cellular cozymase. Almost all the nicotinic acid derivatives resulting from the breakdown of cozymase in Exp. A, Table 7, were found in solution and not in the cells.

Products of cozymase inactivation by streptococci

Nicotinamide moiety Although the specimens of cozymase which were employed in this investigation were not pure, they contained little nicotinic acid which was not in the form of cozymase (Table 1). The bulk of the nicotinic acid of streptococci also appeared to exist as cozymase (Table 7C). When streptococci were caused to react with added cozymase, the cozymase content of the system fell with out a corresponding decrease in its content of nicotinic acid derivatives. This is shown in each experiment of Table 7.

The nicotinic acid estimations of Table 7 were performed after heating with acid under conditions (see experimental section) which other workers have found to liberate nicotinic acid from the majority of its naturally occurring derivatives. The preceding

observations, therefore, do not imply that nicotinic acid itself is formed from cozymase. As nicotinic acid exists in cozymase as its amide, we have sought information on the fate of the amide grouping. Atkin, Schultz, Williams & Frey's (1943) method for distinguishing between nicotinamide and nicotinic acid was found to be an excellent one, and applicable when nicotinic acid was assayed with *Lactobacillus arabinosus*. Solutions were assayed before and after treatment with hypobromite. This converted the amide but not the acid to the amine, which was without growth-promoting activity. Table 7B shows the product from cozymase to behave similarly to nicotinamide in this respect. It also shows the specimen of cozymase which we have employed to contain <0.5% of free nicotinic acid, or of derivatives of nicotinic acid which failed to be inactivated by hypobromite (cf. Schlenk, 1945).

The stability of nicotinamide, when added as such to streptococci, was investigated to see whether it was comparable to that of the product from cozymase. Reaction mixtures similar to that of Exp. A, Table 5, but containing nicotinamide in place of cozymase, were kept at 37° for 90 min. Ammonia was determined by Nessler's reagent after distillation under reduced pressure. Ammonia formation occurred in the absence of nicotinamide at the rate of about 40 $\mu\text{mol}/\text{mg}/\text{hr}$, and no difference was detected in this rate when 4 or 40 μmol of nicotinamide were added. A difference of 10 $\mu\text{mol}/\text{mg}/\text{hr}$ could have been detected. Corresponding quantities of cozymase would have been inactivated at rates of at least 400 $\mu\text{mol}/\text{mg}/\text{hr}$ under the conditions of the reaction. Any hydrolysis of nicotinamide thus appears to be at a rate not exceeding 10% of that of the inactivation of cozymase, in reality, it may be much less.

Loss of V-factor activity The term V factor has been retained as a collective name for a group of

Table 7. *Stability of nicotinamide moiety of cozymase in the presence of streptococci*

(In mixtures A, varying quantities of streptococci R, prepared as described in Table 5, were incubated aerobically with cozymase in 0.017M Na and K phosphates of pH 7.5. At the times indicated, portions of the reaction mixtures were cooled, centrifuged and the cozymase (by apozymase) and nicotinic acid in solution were determined. Mixtures B were similar but determinations were carried out on the whole reaction mixture, including bacteria. Mixtures C were buffered with veronal (0.05M, pH 7.5) and determinations included bacteria.)

Mixture	Cozymase content (μmol)	Nicotinic acid content (μmol)	Nicotinic acid remaining after treatment with hypobromite (μmol)
A Before reaction	57.3	55	—
With 11.4 mg organisms, 10 min.	3.9	57	—
With 11.4 mg organisms, 40 min.	3.6	60	—
With 3.8 mg organisms, 10 min.	23.7	59	—
With 3.8 mg organisms, 40 min.	11.7	61	—
B Organisms and cozymase before reaction	24	26	0.1
Organisms and cozymase after reaction	0.4	27	0.15
Nicotinic acid	—	406	420
Nicotinamide	—	406	1.2
C Organisms and buffer	60	68	—
Cozymase and buffer	102	115	—
Above quantities of organisms, cozymase and buffer after 1 hr at 37°	96	172	—

substances, any one of which will support the growth of *Haemophilus parainfluenzae* under certain conditions described in the experimental section. These substances are known to include coenzymes I and II, a 'desaminocozymase' (Schlenk, Hellström & Euler, 1938) and nicotinamide riboside. The simplest of these is the riboside, and the others are derivatives of it.

When cozymase had been inactivated with respect to the apozymase or muscle systems, we found that it had lost also most of its V factor activity. Moreover, when part only of its activity in the apozymase system had been lost, a similar part of its V-factor activity had disappeared (Table 8).

Isolation of nicotinamide picrolonate. The simplest conclusion from the preceding paragraphs is that the streptococcal reaction with cozymase produces a molecule smaller than nicotinamide riboside, but still containing the nicotinamide part of the molecule. Following a method similar to that of Handler & Klein (1942), who were concerned with the breakdown of cozymase by animal tissues, we have

attempted to isolate nicotinamide itself from the products of streptococcal inactivation of cozymase.

Table 7 shows that the product from added cozymase which carries the nicotinic acid portion of the molecule remains in solution and not associated with the streptococci. The reaction mixture of Table 5A was accordingly kept at 37° until samples showed all but 3% of its cozymase to be inactivated. It was then centrifuged and the organisms washed twice with 8 ml. of water. The combined solutions were acidified to pH 3 with 5N H₂SO₄, and Ag₂SO₄ (1.7 mmol) added until no more precipitate formed, this was separated. The solution was freed from Ag⁺ by H₂S, and after removing the latter by N₂, it was taken to pH 9 with Ba(OH)₂, N₂ again passed, then adjusted to pH 7-7.5 with H₂SO₄, BaSO₄ removed and washed, the combined solutions evaporated to dryness under reduced pressure and the residue left *in vacuo* with CaCl₂ overnight. It was extracted by refluxing 3 times successively with 8 ml. of absolute ethanol, the ethanol solutions evaporated to dryness and the residue re-extracted 3 times with 2 ml. of absolute ethanol. Determination of nicotinic acid showed that all the acid had not been extracted by these procedures, the residues were accordingly re-extracted in the same way, when >90% of the materials of

Table 8 *Loss of V-factor activity with inactivation of cozymase*

(Smaller quantities of streptococci R were prepared as described in Table 5. In Exp. A, determinations were carried out on reaction solutions after centrifuging free from streptococci. In B, the separations indicated were carried out. Reactions were anaerobic.)

Reaction mixture (cozymase in 2.5 ml. with)	Cozymase determined with apozymase (μmol/ml)	V factor activity (as μmol cozymase/ml)
Exp. A		
(1) 0.015M Phosphates, pH 6	17.3	17.8
(2) As (1), with streptococci (6.5 mg), 40 min. at 37°	8.2	7.8
(3) 0.015M Phosphates, pH 7, with streptococci (6.5 mg), 10 min. at 37°	13.9	12.2
(4) As (3), for 40 min.	1.7	1.7
Exp. B		
(1) 0.011M Phosphates, pH 7.6	11.0	12.0
(2) As (1), with streptococci (8.3 mg), 40 min. at 38°, determination in solution	0.8	1.0
(3) Cells from reaction (2)	13.0	17.4
(4) Streptococci (8.3 mg), before reaction	16.9	17.3

Table 9 *Nicotinamide from the streptococcal breakdown of cozymase*

(Exp. B is that of Table 5A, where further details are given. The cozymase of specimens which were taken to follow the course of the reaction has been subtracted from that added. A similar reaction mixture was employed in Exp. A, but with a different batch of organisms and with nicotinamide in place of cozymase. Nicotinic acid picrolonate depresses the melting point of that of nicotinamide.)

	Experiment	
	A	B
Added	Nicotinamide, 52 μmol	Cozymase, 31.5 μmol
Isolated as fairly pure picrolonate of nicotinamide, m.p. 214-217°, not lowered by mixing with the pure substance (μmol)	17	16
Isolated as less pure material, m.p. 208° or greater, not lowered by mixing with nicotinamide picrolonate (μmol)	16	5
Picrolonic acid content of purer product, by titration with methylene blue (Bolliger, 1939, theory for nicotinamide 68.5%) (%)	69	70
Yield of nicotinic acid from purer picrolonate, determined by <i>Lactobacillus arabinosus</i> (theory for nicotinamide picrolonate, 31%) (%)	—	28

nicotinic acid activity were obtained in ethanolic solution. This solution was evaporated to c 0.5 and 2.5 ml of 1% (w/v) picrolonic acid in 50% aqueous ethanol added, and the solution kept cold. Yellow crystals separated, further material was obtained from the mother liquors by evaporation and addition of more picrolonic acid. By one recrystallization from aqueous ethanol the fractions of Table 9, Exp B, were obtained. These indicate that the bulk of the product which has nicotinic acid activity is nicotinamide, and that its yield and purity are comparable to that of nicotinamide isolated from a similar reaction mixture to which it had been added as such, in place of cozymase (Exp A). Exp A was carried out first to serve as a model in the isolation of the product from cozymase. The data of Table 9 afford independent evidence for the stability of nicotinamide under conditions in which cozymase is inactivated.

splitting analogous to that in the liberation of nicotinamide from cozymase would lead to the formation of nicotinic acid itself, small quantities of which can be detected. Any reaction in trigonelline brought about by streptococci, however, aerobically or anaerobically, was of <1/1000 of the rate of the organisms' reaction with cozymase.

Inactivation of cozymase by Haemophilus parainfluenzae

The change brought about by this organism appears similar to that caused by streptococci. The course of loss of cozymase activity from solutions initially about $5 \times 10^{-5} M$ was paralleled closely by loss of V factor activity (Table 10). The nicotinic acid content of a similar reaction mixture did not fall

Table 10 Breakdown of cozymase by *Haemophilus parainfluenzae*

(Strain 4101 was employed and grown in the assay medium with the addition of 3% of yeast extract, initial concentration of cozymase in the medium, 2 μg /ml. The organisms were washed twice with, and suspended in, 0.9% NaCl. The quantity of cozymase indicated was added with phosphates (to 0.03M) at pH 7. Incubation was aerobic at 37°. Determinations refer to the whole mixture, including bacteria. Cozymase itself remained unchanged in the absence of bacteria.)

Batch of organisms, time of growth, quantity used in reaction (dry wt.)	Time of reaction (min)	Initial cozymase ($\mu\mu\text{mol}/2.5 \text{ ml.}$)	% remaining of		
			Cozymase by apozymase	V factor (as cozymase)	Nicotinic acid
A, 40 hr, 4.2 mg	0	120	(100)	(100)	—
	90	120	56	53	—
	180	120	19	19.5	—
	330	120	4	7	—
B, 46 hr, 10.5 mg	0	129	(100)	—	(100)
	90	129	14.4	—	97
	180	129	<3	—	100
	330	129	<3	—	104

Other changes during inactivation of cozymase, other substrates. We have found changes in the phosphate of streptococcal suspensions to be too great to obtain dependable information on the possible liberation of inorganic phosphate from cozymase during its inactivation by the organisms.

The possible production of acid during the reaction was examined by following the evolution of CO_2 from a medium containing 0.038M bicarbonate in equilibrium with 5% CO_2 in N_2 . During the inactivation of 1.7 μmol cozymase 2.35 μmol CO_2 were evolved from a vessel containing streptococci, and 2.43 μmol from one also containing the cozymase. A similar reaction mixture containing yeast nucleic acid (3 mg/ml), instead of cozymase, yielded CO_2 in large excess of a control without substrate, and corresponding to acid formation at the rate of 300 $\mu\text{mol}/\text{mg}/\text{hr}$. Thymus nucleic acid also reacted, any reaction with yeast adenylic acid and guanylic acid was much slower. The yeast nucleic acid was purified according to Gulland & Jackson (1938), and acid formation from it was not inhibited by 0.08M azide, which reduced glycolysis to about 35% of its normal value. The streptococci thus appear to contain a nuclease, but their reaction with cozymase is of a different type from that with nucleic acids.

Pyridine N linked to carbon is found in other natural products. Of these, trigonelline was examined, since a

during a period at least twice as long as that which sufficed to inactivate almost all its cozymase. Breakdown therefore does not appear to lead to accumulation of nicotinamide riboside, but it does not extend beyond nicotinic acid.

Effect of nicotinamide on the streptococcal breakdown of cozymase synthesis of cozymase

The effect of nicotinamide on the streptococcal breakdown of cozymase was examined since nicotinamide is a product of the reaction, and since it has been found to inhibit the analogous breakdown in animal tissues (Mann & Quastel, 1941, Handler & Klem, 1942). Retardation of the streptococcal reaction was found, but only with extremely high concentrations of nicotinamide. The retardation by 0.1M nicotinamide (2000 times the substrate concentration) was of the order of 50% (Table 11). Nicotinic acid salts had little or no action at 0.033M.

Other products of cozymase breakdown by streptococci remain unknown, but we have attempted to obtain a synthesis of the material from nicotinamide and yeast adenylic acid. Table 11 shows that synthesis did not occur from these compounds alone,

Table 11 *Nicotinamide in the breakdown and synthesis of cozymase*
(*Streptococci* (strain R) were used on their first day of growth, reactions were aerobic and at 37°)

Batch of organisms, conditions of reaction, time of growth		Reaction mixture	Cozymase remaining (%)
	Substances added	Initial cozymase	
A, pH 7.5, 60 min	0	$4.8 \times 10^{-5} M$	14
	Nicotinamide, 0.1 M	$4.8 \times 10^{-5} M$	52
	Nicotinamide, 0.033 M	$4.8 \times 10^{-5} M$	22
	Na nicotinate, 0.033 M	$4.8 \times 10^{-5} M$	17
B, pH 7.5, 40 min	0	$5 \times 10^{-4} M$	26
	Nicotinamide, 0.055 M	$5 \times 10^{-4} M$	47
	Nicotinamide, 0.028 M	$5 \times 10^{-4} M$	29
			Change in cozymase (μ mol)
C, pH 7.5, 40 min	0	22 μ mol (in cells)	—
	{Adenylic acid, 0.0033 M Nicotinic acid, 0.0033 M or both	22 μ mol (in cells)	<1
C, pH 7.5, 10 min	{Adenylic acid, 0.0033 M Nicotinic acid, 0.0033 M and glucose 0.033 M	22 μ mol (in cells)	+12.4
	As above	22 μ mol (in cells)	+26

but did take place when glucose also was added. In a similar reaction mixture the course of reaction was followed in more detail and the cozymase was found to increase at the rate of 20 μmol/mg dry wt/hr during the first 50 min.

DISCUSSION

Streptococcal breakdown of cozymase Inactivation of cozymase has been shown to be a very general reaction in bacteria. The reaction in streptococci reached surprisingly high velocities. With cozymase at $4 \times 10^{-4} M$, it was decomposed at rates about 400 μmol/mg of organisms/hr, equivalent to the decomposition during 1 hr of a quantity of cozymase one quarter of the bacterial dry weight. Studies of the essentials for microbial growth, and the development of microbial methods for estimating vitamin-like compounds, have tended to give the impression that bacteria normally react with only small quantities of such compounds. This is true only in one sense. Thus, the minimal concentration of nicotinic acid or its derivatives normally needed for maximal bacterial growth is less than $10^{-7} M$. This value holds also for the streptococci studied here, though the quantity of cozymase in their cells was of the order of 5–10 μmol/mg dry wt (Tables 2 and 6). The internal concentration of cozymase, assuming a water content of 80%, was therefore about $1-2 \times 10^{-3} M$, or about 0.1% of the cell weight. These are not exceptional values, cozymase contents reported for yeast (cf. Sumner, Krishnan & Sisler, 1946) correspond to internal concentrations of $7 \times 10^{-4} M$. The coenzymes of red blood cells, normally of c. $10^{-4} M$, can be raised to $3 \times 10^{-4} M$ by

feeding nicotinic acid (Handler & Kohn, 1943). The latter values are for substances of V-factor activity, presumably representing the sum of coenzymes I and II. Thus, although the rapid decomposition of cozymase by streptococci was observed with concentrations greater than $10^{-4} M$, cozymase frequently occurs naturally at such concentrations.

It is therefore feasible that the enzymic activity concerned may play a part in the normal economy of the cells, even though substrate saturation is not attained at a cozymase concentration of $4 \times 10^{-4} M$. It is presumably of advantage to an organism to be able to interconvert the naturally occurring nicotinic acid derivatives. An alternative possibility which we considered, but now think unlikely, was that the decomposition of cozymase might occur by the action of a nucleotidase, and although the streptococci produced acid from nucleic acids, acid from cozymase was not formed at a comparable rate. It is certain also that nicotinamide is produced as such from cozymase, and that this involves breakage of the molecule between the pyridine N and the C₁ of ribose. This linkage is known to occur naturally only in the two coenzymes. The somewhat similar link between pyridine N and —CH₂ in trigonelline was split, if at all, at less than 1/1000 of the rate at which cozymase was inactivated. Liberation of nicotinamide from cozymase may be preceded or followed by changes in the remainder of the cozymase molecule. Our present experiments do not give much information on this subject, except that nicotinamide riboside did not accumulate during the breakdown of cozymase. Breakdown of cozymase by acids or alkalis also liberates nicotinamide as such (Schlenk, 1943).

Synthesis of cozymase by streptococci Streptococci were able to synthesize cozymase in spite of the rapid breakdown of added cozymase. This is implied in the finding that cells grown in presence of nicotinic acid contained cozymase, as also did non proliferating suspensions of cells, and it seems that the cozymase initially associated with streptococcal cells was not decomposed so rapidly as the added material. An enzyme inactivating cozymase was not, however, liberated to solution by the streptococci, nor easily extracted from them, and decomposition of cozymase may always be preceded by its assimilation by the cells (cf Table 6). The observed rate of accumulation of cozymase under conditions permitting its synthesis is therefore likely to be less than the true rate of synthesis.

The maximum rate of accumulation of synthesized cozymase which was observed was 20 μmol / mg dry wt / hr. The streptococci used grew, when under satisfactory conditions, with generation times of 20–30 min. The rate of reaction (Q) in μmol / mg / generation time is therefore 6.7–10. As indicated by McIlwain, Roper & Hughes (1948), this means that the cells produced by such growth could have a quantity of cellular cozymase of $q = Q \log_2$, or in this case of 4–6 μmol / mg dry wt. This is about the cell content actually observed (Table 2).

Breakdown of cozymase by other organisms Findings with respect to the breakdown of cozymase in bacteria and in animal tissues are similar, though in neither are they very extensive. Brain suspensions, like the streptococci, liberated nicotinamide (Handler & Klein, 1942). *Haemophilus parainfluenzae* produced either nicotinic acid or nicotinamide. The reaction in animal tissues, as in bacteria, was rapid. The breakdown by rat brain, and probably by cobra venom, was inhibited by nicotinamide (Mann & Quastel, 1941), and we found a lesser breakdown by streptococci in the presence of nicotinamide, though high concentrations of this were required.

The bacteria which we found to inactivate cozymase represent many taxonomic groups: streptococci, staphylococci, *Escherichia*, *Proteus* and *Haemophilus*. They are also very varied in their reactions to nicotinic acid and its derivatives in growth. *Escherichia coli* not requiring such addition for growth, *Proteus morganii* and the cocci requiring nicotinic acid, and *Haemophilus parainfluenzae* requiring nicotinamide riboside. For this reason, breakdown of cozymase by *H. parainfluenzae* has a significance which is different from that of the breakdown by the other organisms. Reaction with

cozymase by *H. parainfluenzae* was at about the same rate as by the other bacteria, but whereas these were capable of resynthesizing cozymase from the products of its breakdown, *H. parainfluenzae* could not do so. It thus inactivates rapidly a compound which is indispensable to its growth. This situation, which is presumably of ecological importance, has parallels in the behaviour of other organisms to other growth essentials (McIlwain, 1947).

One reason for our study of the breakdown of cozymase by *H. parainfluenzae* was as follows. Lwoff & Lwoff (1937) concluded from indirect evidence that added coenzymes I and II could be interconverted by this organism, though after assimilation such interconversion was not possible. Production from added cozymase of a simpler substance of V factor activity would have provided a mechanism for the interconversion, but we were unable to demonstrate the production of such a substance.

SUMMARY

1 Cozymase at concentrations of about 10^{-4} – 10^{-5}M was inactivated by streptococci, staphylococci, *Escherichia coli*, *Proteus morganii* and *Haemophilus parainfluenzae* (but not by *Lactobacillus arabinosus* 17–5) at rates of 5–30 μmol / mg dry wt of cells/hr at pH 6–7.5 and 37°.

2 Streptococcal cells have been found to be about 10^{-3}M with respect to cozymase, which was the form in which most of their nicotinic acid existed. Streptococci inactivated $4 \times 10^{-4} \text{M}$ cozymase at the rate of 400 μmol / mg / hr, and so could inactivate their own dry weight of the substance in 4 hr.

3 Nicotinamide was produced from cozymase during its inactivation by streptococci. A nicotinamide derivative of V-factor activity, such as the riboside, did not accumulate. The breakdown by *Haemophilus parainfluenzae* was similar.

4 The breakdown in several bacteria was not greatly influenced by air (but was sometimes greater anaerobically), or glucose (this sometimes retarded it), and was still rapid at 10°.

5 Streptococci were able to synthesize cozymase from nicotinic acid and adenylic acid in the presence of glucose, rates of +20 μmol / mg dry wt / hr were observed, which were about those required to account for the observed cell content of the substance.

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The Amylose Content of the Starch Present in the Growing Potato Tuber

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During the last few years it has been shown that starch consists of two components amylose, which is a linear polymer and comparable with cellulose, and amylopectin, which is a branched chain polymer comparable with glycogen. The advances in this field have been greatly assisted by the development of the potentiometric iodine titration method for estimating the percentage of amylose in starch (Bates, French & Rundle, 1943; Hudson, Schoch & Wilson, 1943). By use of this procedure the amylose content of many starches has been determined, and starches have been examined in which the amylose content varies from less than 1% in the waxy starches to over 50% in certain varieties of pea starch (Bates *et al* 1943; Hilbert & MacMasters, 1946). The published values of amylose contents for starches of similar botanical origin, e.g. maize, sometimes differ and this suggests that the amylose content is not necessarily a constant property of the starch from any given type of botanical species. Furthermore, by use of the newer methods for end-

group determination, and with a knowledge of the amylose content of a starch, it is now possible without separation of the components to determine the proportion of glucose residues present in the amylopectin fraction of the starch as terminal groups (Brown, Halsall, Hirst & Jones, 1948). For example, the sample of rice starch which was examined by Hirst & Young (1939), who used the methylation technique, has now been shown to contain 12% of amylose, and hence we have present in the amylopectin fraction one non-reducing terminal group/26 glucose residues. In other samples of rice starch, however, an amylose content of 14–15% (Brown *et al* 1948) has been found, and the amylopectin fraction has been shown to contain one non-reducing terminal residue/20 glucose residues. This indicates that different varieties of rice starch do not all contain the same type of amylopectin. It will be recalled that Meyer & Heinrich (1942) have claimed that the starches extracted respectively from potato tubers, leaves and shoots, contain different pro-

portions of amylose and amylopectin, the shoots in particular having a high proportion (approx 46 %) of the amylose component

In view of this evidence it was considered desirable to investigate the amylose content and the ratio of terminal residues to non terminal residues present in the starch of two different varieties of the same botanical species throughout their period of growth. We therefore decided to examine at frequent intervals during the growing season the starch present in the tubers of two varieties of potatoes, one an early and the other a main crop variety

EXPERIMENTAL

Growing of potatoes The potatoes were grown from virus free Golden Wonder and King Edward 'seed', which was very kindly given to us by the Seale Hayne Agricultural College.

Isolation of the starch The potatoes (c 600 g) were washed to remove soil, scraped to remove the skin and cut into small pieces, which were broken up in an ordinary mincing machine. The coarse mash thus obtained was filtered and then ground up with a little water in a mill. The resulting fine slush was filtered, and allowed to stand for some hours in 1 % sodium sulphite solution with intermittent shaking. The solid matter was then allowed to settle out and the supernatant liquid, which was usually dark in colour, was decanted. This process was repeated two or three times until the sulphite solution no longer developed any colour. The solid matter was then suspended in water and filtered through coarse muslin, when fibrous material remained on the cloth whilst the starch granules passed through. They were allowed to settle and then washed by decantation with distilled water, the water being poured off before complete sedimentation had occurred. This procedure ensured that any of the less dense fibrous

material which had come through the filter was removed from the starch. Finally, the starch was filtered off on a hardened smooth filter paper to avoid contaminating it with cellulose fibres, washed first with water and then with methanol, and dried under diminished pressure. The yield of starch was of the order of 40–60 g. More starch of a poorer quality (e.g. slightly discoloured) could be obtained by repeating the above extraction process with the fibrous material left on the muslin filter.

Investigation of the starches All starch samples were first defatted by treatment in a Soxhlet apparatus for 18 hr with a constant boiling mixture of dioxan (80 %) and water (20 %) (Schoch, 1942, 1946). The sulphated ash content of the fat-free starch was determined.

The amylose contents of the starch samples were determined by means of the iodine potentiometric method of Bates *et al* (1943) as modified by Hudson *et al* (1943). In our determinations a Cambridge potentiometer reading to 0.1 mV was used in conjunction with a mirror galvanometer. In the calculation of the amylose content the amylose was assumed to take up 21.5 g iodine/100 g amylose (Higgin botham & Morrison, 1947). In many of the previous publications on this subject a lower figure (c 19.2 g iodine/100 g amylose) has been used for the calculation of amylose content, and the percentages of amylose quoted will therefore be a little high. It is probable that some of the work which has been reported as having been carried out with pure amylose refers in fact to samples of amylose containing 5–10 % amylopectin.

In addition to determining the amylose content certain other properties were investigated with certain of the starch samples. With these samples nitrogen estimations (micro Dumas) were carried out to see whether any appreciable amount of protein was being isolated along with the starch. The optical rotations of these samples in N NaOH were also determined, and finally the samples were oxidized by KIO₄, and from the amounts of formic acid produced the number of glucose residues containing one non reducing terminal

Table 1 *Properties of starches from two varieties of potatoes*

Date of collecting potatoes	Ash content (%)	N (%)	$[\alpha]_D^{20}$ in N NaOH	Amylose* (%)	A†	B†
Golden Wonder potatoes						
10 vii. 46	0.8	< 0.1	+160°	18	32	26
16 vii. 46	1.2	—	—	18	—	—
24 vii. 46	0.8	—	—	19	—	—
31 vii. 46	0.9	—	—	19	—	—
7 viii. 46	1.2	< 0.1	+169°	18	33	27
14 viii. 46	0.5	—	—	16	—	—
20 viii. 46	0.4	—	—	16	—	—
5 ix. 46	0.7	—	—	16	—	—
10 ix. 46	0.9	< 0.1	+160°	16	32	27
King Edward potatoes						
31 vii. 46	0.6	< 0.1	+159°	16	32	27
7 viii. 46	1.1	—	—	18	—	—
14 viii. 46	0.7	—	—	15	—	—
20 viii. 46	0.6	< 0.1	+159°	15	33	28
5 ix. 46	0.8	—	—	16	—	—
10 ix. 46	0.5	—	—	16	—	—
24 ix. 46	0.8	0.4	+161°	17	33	27

* The estimated error in the figures of amylose contents is $\pm 1\%$, e.g. $18 \pm 1\%$ amylose

† Col. A gives the number of glucose residues/one non reducing terminal residue in the whole starch, and col. B the corresponding figure for the amylopectin fraction. The estimated error in the end group determinations is ± 2 in 30 glucose residues, e.g. 32 ± 2 glucose residues/one non reducing terminal residue

residue was calculated for the various samples. From these figures and the percentages of amylose present the number of glucose residues containing one non reducing terminal residue in the amylopectin fraction was estimated. The results of the analyses and calculations are given in Table 1.

DISCUSSION

The figures for the amylose content of the starch for King Edward potatoes varied between 15 and 18 %, but showed no definite upward or downward trend. In the case of the starch for Golden Wonder potatoes the upper and lower figures were 19 and 16 %, respectively, and the lower values were observed at the later period of growth. The variation is, however, only just outside the estimated range of experimental error, and it is not possible at this stage to decide whether or not it is significant. For neither of these starches can it be said, on the basis of the present data, that any noticeable change in the proportion of amylose and amylopectin occurs during the period of growth. Furthermore, the

results indicate that the amylopectins present in the two varieties of potato are identical, and belong to that group of amylopectins which contain some 24-26 glucose residues/terminal group. This proportion of end groups is the same as that found also in other samples of potato starch (Brown *et al.* 1948).

SUMMARY

1. Samples of the starches present in the tubers of two varieties of potato, Golden Wonder and King Edward, have been isolated at intervals throughout the period of growth of the plants.

2. No significant variation in the properties of amylose (c. 17 %) and amylopectin (c. 83 %) was observed during the period of growth.

3. In the amylopectin portion of the starches there was one terminal glucose residue for every 24-26 glucose residues, and the amylopectin for the two varieties of potatoes appeared to be identical.

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The Sorption of DDT and its Analogues by Chitin

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On the basis of a general correlation between the possession of a chitinous cuticle and sensitivity to DDT poisoning, and the fact that chitin will sorb DDT from colloidal suspension, Richards & Cutkomp (1946) put forward the hypothesis that the chitin of the cuticle serves to concentrate DDT selectively from the media with which the insects are treated, and so to give a higher concentration inside the animal.* If this is actually the case then it is reasonable to expect that the toxicity of compounds

similar to DDT will be affected in some degree by the extent to which they are sorbed by chitin, and that, in making comparisons of their potencies account must be taken of the degree of concentration of these substances in the insect by the sorptive action of the chitinous cuticle.

Experiments were therefore carried out to observe the sorption by chitin of a number of DDT analogues from colloidal suspensions. In order to do this it was necessary to devise a method for estimating the DDT or its analogues sorbed from colloidal suspension, and also to obtain a general picture of the effect of time, concentration and amount of chitin present on the amount of DDT sorbed.

* These authors say that DDT is adsorbed by chitin. This implies a surface action and since no proof is offered as to the mechanism of the process the term 'sorption' has been used throughout this paper since this word does not imply any particular mechanism.

Under the experimental conditions described here, it is possible that DDT is mechanically retained by chitin. The retention of DDT by chitin was therefore compared with that by fine sand, which would also retain DDT from colloidal suspension if a mechanical action only were involved.

In an attempt to obtain some information on the mode of sorption of DDT by chitin, the chitin was replaced by other materials, viz. cellulose, because of its similar structure to chitin, and wool representing a proteinous group of materials. Also, the sorption by chitin of different particle sizes was examined, but unfortunately it was not possible to measure the particle size of the chitin owing to the irregular size of the laminar particles.

The work described has been of a preliminary and exploratory nature upon which further investigations might be based, and it was not intended as an exhaustive examination of the problem of sorption of DDT and its analogues by chitin. Sufficient information was sought to decide whether or not the great difference in the toxicities of DDT analogues to insects might possibly be explained by differences in the rate or extent to which they are sorbed by chitin. Such differences were not observed, and experiments with further groups of analogues were not continued since it is unlikely that useful information would be obtained in this way. Further investigation on the effect of temperature on the sorption of DDT by chitin might, however, throw some light on the influence of temperature on the toxic action of DDT, and the work is to be continued with this end in view.

METHODS

Preparation of colloidal suspensions of DDT and its analogues. Colloidal suspensions of DDT may be prepared by the exchange of solvents method. Relatively stable suspensions, from which the disperse phase is not deposited for 24 hr. or more, may be prepared by rapidly diluting with water ethanolic solutions of DDT or its analogues. This can perhaps be best effected by running the solution from a funnel into a well stirred volume of water. Although the method allows of considerable control of the rate of dilution of the ethanolic solution, it presents difficulties when the amount of ethanol used is barely sufficient to dissolve at room temperature all the DDT or analogue present. In this case considerable quantities of solute are likely to crystallize out on the sides of the funnel, when the solvent evaporates from the large surface presented as the solution drains from the funnel. Moreover, in the circumstances under consideration, it is not possible to redissolve the deposited solute with more solvent. The use of hot ethanolic solution is also not practicable, since the greater evaporation from the hot solvent, combined with the rapid cooling by the glassware, tends to increase the amount of crystalline material separating out. It is, however, possible to prepare colloidal suspensions from ethanolic solutions of DDT analogues, saturated at room temperature, by adding water very rapidly to the vigorously stirred ethanolic solution. In this

way separation of DDT analogue from the ethanolic solution before dilution with water is avoided.

In order to facilitate the rapid dilution of a small amount (5-10 ml.) of solution with a large bulk (100 ml.) of water, a special apparatus, made by cutting a 500 ml. round bottomed flask through its maximum diameter, was constructed. The neck of the flask was then drawn out and sealed off to give a goblet-shaped vessel. To prepare a colloidal suspension, 10 ml. of ethanolic solution of DDT analogue were placed in the small cup formed by the sealed off neck portion of the flask. The ethanolic solution was then stirred vigorously with a spiral glass stirrer driven by a small electric motor, and 90 ml. of distilled water were added as rapidly as possible from a measuring cylinder. An almost instantaneous dilution was obtained. A further 100 ml. of water were then added as rapidly as possible to give a colloidal solution of DDT analogue in 5% ethanol in water.

Sorption of DDT analogues on to chitin. The same general procedure was used throughout. A colloidal suspension of DDT, or one of its analogues under consideration, was freshly prepared and samples were measured out into conical flasks. Weighed portions of chitin (from lobster shell) were then added to the contents of some of these flasks, the remainder being used as blanks to correct for any analogue not in colloidal suspension. The flasks were then kept gently agitated on a mechanical shaking machine for the desired time.

For simplicity, the treatment of an individual flask and its contents will now be described, since from this point onwards the tests and blanks were treated in exactly the same way. After sorption had taken place for the desired period, the contents of the flask were filtered through a no. 3 sintered glass crucible, and any solids which remained in the flask were washed into the filter with distilled water. The washing of the filter with distilled water was continued until it was free from colloidal suspension. The DDT analogue retained by the filter was then estimated. Tests were also carried out with other materials instead of chitin.

Estimation of DDT or analogue sorbed by chitin. Of the methods available for the determination of DDT, only those based on the determination of either all or a definite part of the halogen of the molecule are directly applicable to DDT analogues. Other methods of estimating DDT are based on colour reactions, which may or may not be satisfactory for the estimation of its analogues. Therefore, since the removal of one molecule of HCl by alkali occurs very readily with both DDT and its analogues, the following method of estimating them was used in preference to any other method. The sintered glass crucible, through which the colloidal solution of DDT or one of its analogues had been filtered, was allowed to drain, and any water remaining in the underside was removed with a piece of filter paper. The crucible was then fitted into a dry holder held by a rubber bung in a Buchner flask, which contained a small test tube to collect the 5 ml. of acetone were used to dissolve any DDT or analogue retained by the crucible. The crucible holder was then washed with a little acetone, and the contents of the test tube were transferred to a 100 ml. stoppered conical flask, with further washing with acetone. The crucible was again washed with 5 ml. of acetone, which was collected in the test tube and transferred to the conical flask. The crucible was then washed successively with 0.5N ethanolic NaOH (1 ml.), ethanol (2 ml.) and acetone (5 ml.), and all washings collected in the test tube and finally transferred to the conical flask.

The solution in the conical flask was then evaporated to dryness on a water bath, not only to reduce the bulk of liquid, but also to ensure complete hydrolysis of the DDT or analogue present. In the meantime, the crucible had been washed with 5 ml. of distilled water, which were added to the residue in the conical flask. The liberated halogen was then estimated by the Volhard method. The contents of the conical flask were acidified with approx. 8N HNO₃ (0.5 ml.)

and an excess of 0.01N-AgNO₃ added. Nitrobenzene (1 ml.) was added, and the excess silver was titrated with standard 0.01N NH₄CNS using one or two drops of saturated ferrous ammonium sulphate solution as indicator.

RESULTS

The results are presented in Tables 1-6.

Table 1 *The sorption of DDT by varying amounts of chitin*

(A colloidal suspension containing 0.05% DDT in 5% ethanol in water was prepared. Portions (20 ml.) of this suspension were agitated for 80 min. with various quantities of chitin, after which the amount of DDT sorbed by the chitin was estimated.)

Date	Chitin (g)	DDT sorbed (corrected for blanks) (mg)	DDT sorbed by chitin (mg/g)	DDT in blanks with no chitin (mg)
16 vii 46	0.10	2.5	25	1.1
	0.20	2.6	13	0.6
	0.30	6.1	20	
	0.40	7.6	19	Mean 0.9
17 vii 46	0.20	2.3	11	0.0
	0.30	4.1	14	1.0
	0.40	7.0	18	Mean 0.5
26 ix 46	0.11	2.0	18	0.2
	0.21	3.3	16	0.6
	0.31	4.8	15	
	0.39	6.6	17	Mean 0.4
	0.50	8.0	16	—

Sand used instead of chitin (cf. 17 vii 46)

Sand (g)	DDT retained (not corrected for blanks) (mg)
0.20	0.6
0.30	0.6
0.40	0.7

Table 2 *The sorption of DDT by chitin during various periods of time*

(Portions (0.3 g.) of chitin were added to 20 ml. portions of 0.05% DDT colloidal suspension in 5% ethanol in water. The mixtures were agitated for various lengths of time, after which the amount of DDT sorbed by chitin was determined.)

Date	Time of sorption (min)	Chitin (g)	DDT sorbed (corrected for blanks) (mg)	DDT sorbed by chitin (mg/g)	DDT in blanks with no chitin (mg)
27 ix 46	30	0.31	2.9	9	0.7
	60	0.31	4.5	15	0.2
	120	0.30	5.5	19	0.1
	240	0.31	6.5	21	1.4
					Mean 0.6
30 ix 46	30	0.31	4.2	14	0.2
	60	0.31	5.6	18	0.1
	120	0.30	8.7	29	0.9
	180	0.30	9.0	30	0.1
	240	0.30	8.6	29	0.2
					Mean 0.5

Table 3 *The effect of varying the concentration of DDT on its sorption by chitin*

(Portions (20 ml) of 0.5% DDT colloidal suspension in 5% ethanol in water were diluted with 5% ethanol to give various concentrations of DDT. To samples of these various dilutions, chosen to contain 10 mg of DDT, portions of 0.3 g of chitin were added and agitated for a period of 95 min.)

Date	DDT concentration (%)	Chitin (g)	DDT sorbed (corrected for blanks) (mg)	DDT sorbed by chitin (mg/g)	DDT in blanks with no chitin (mg)
2 x 46	0.05	0.31	5.8	19	0.2
	0.025	0.31	2.3	7	0.7
					Mean 0.5
3 x 46	0.05	0.32	6.7	21	0.4
	0.04	0.30	4.2	14	
	0.025	0.31	—	—	1.0
					Mean 0.7
4 x 46	0.05	0.31	6.0	19	0.2
	0.04	0.31	4.6	15	
	0.033	0.30	3.4	11	
	0.025	0.30	1.6	5	
	0.013	0.31	1.8	3	1.1
					Mean 0.6

Table 4 *Sorption of DDT analogues by chitin*

(The sorption by chitin of the four symmetrically para-substituted halogen derivatives of 1,1,1 trichloro 2,2 diphenyl-ethane from colloidal suspension in 5% ethanol in water was examined. Samples (40 ml) of colloidal suspensions of the analogues, each containing 18.6×10^{-6} mol of analogue, were agitated with portions (0.30 g) of chitin for various lengths of time, after which the amount of analogue sorbed by the chitin was estimated.)

Date	Time (min)	Chitin (g)	Analogue sorbed (corrected for blank)		Analogue in blank using no chitin (mg)
			(mg)	(mol. $\times 10^{-6}$)	
1 1 1 Trichloro 2 2 bis(4' fluorophenyl)ethane, 0.0151% suspension					
23 x. 46	60	0.30	1.1	3.4	0.6
	115	0.30	1.1	3.4	
	150	0.29	2.0	6.2	
	195	0.30	2.4	7.5	0.7
	240	0.30	3.2	9.9	
	300	0.30	3.0	9.3	
	360	0.30	3.1	9.6	1.1
					Mean 0.8
1 1 1 Trichloro 2 2 bis(4' chlorophenyl)ethane, 0.0165% suspension					
18 x. 46	30	0.30	1.0	2.8	0.3
	60	0.30	1.4	3.9	
	90	0.29	1.6	4.5	
	150	0.30	2.0	5.6	
	180	0.30	2.3	6.5	
	210	0.30	2.9	8.2	
	240	0.30	2.5	7.0	
	270	0.30	2.2	6.2	0.1
				Mean 0.2	
25 x. 46	60	0.30	0.9	2.5	0.5
	115	0.30	1.2	3.4	
	150	0.30	2.8	7.9	
	195	0.30	2.9	8.2	0.3
	240	0.30	3.0	8.5	
	300	0.30	4.2	11.8	0.0
					Mean 0.3

* 18.6×10^{-6} mol of DDT or its analogue in 40 ml of 5% ethanol in water was chosen so that these results would be comparable with the earlier experiments on the sorption of DDT and its analogues. It corresponds to 0.025% (w/v) suspension of the iodo analogue.

Table 4 (cont)

Date	Time (min)	Chitin (g)	Analogue sorbed (corrected for blank)		Analogue in blank using no chitin (mg)
			(mg)	(mol $\times 10^{-6}$)	
1 1 1 Trichloro 2 2 bis(4' bromophenyl)ethane, 0.0206% suspension					
21 x 46	60	0.30	1.7	3.8	1.0
	115	0.30	2.8	6.3	
	150	0.29	4.8	10.8	0.7
	195	0.30	3.8	8.6	
	240	0.30	4.4	9.9	
	300	0.29	5.2	11.7	
	360	0.29	5.6	12.6	0.0
					Mean 0.6
1 1 1 Trichloro 2 2 bis(4' iodophenyl)ethane, 0.0250% suspension					
11 x 46	30	0.31	0.4	0.7	2.9
	60	0.30	1.5	2.8	1.2
	120	0.31	4.1	7.6	2.2
	180	0.30	6.4	11.9	0.0
	240	0.31	5.7	10.6	0.2
					Mean 1.3

(Amount of DDT sorbed in 4.5 hr by 0.3 g of chitin from a colloidal suspension containing 18.6×10^{-6} mol. of analogue in 40 ml of 5% ethanol in water)

Amount sorbed (mol. $\times 10^{-6}$, corrected for blank) Blank (mol $\times 10^{-6}$)

1 1 1 Trichloro 2 2 bis(4' fluorophenyl)ethane			
11.8		2.7	
10.9		4.6	
12.0		3.3	
11.7		4.1	
12.4			
Mean 11.8 \pm 0.6		3.7	
1 1 1 Trichloro 2 2 bis(4'-chlorophenyl)ethane			
12.4		2.1	
11.3		2.3	
11.4		1.7	
9.4		2.4	
9.2			
Mean 10.7 \pm 1.4		2.1	
1 1 1 Trichloro 2 2 bis(4' bromophenyl)ethane			
11.5	13.0	1.7	0.2
11.6	14.2	2.3	0.0
15.7	13.9	1.1	
14.7			
Mean 13.4 \pm 2.2	13.7 \pm 0.6	1.9	0.1
1 1 1 Trichloro 2 2 bis(4' iodophenyl)ethane			
13.5		1.9	
14.2		1.5	
14.7		0.9	
15.5		2.0	
13.0			
Mean 14.2 \pm 1.0		1.6	
1 1 1 Trichloro 2 2-diphenylethane			
10.9		3.2	
9.3		2.3	
12.1			
Mean 10.8		2.8	
1 1 1-Trichloro 2 2 bis(4'-ethoxyphenyl)ethane			
7.3		0.8	
10.3		0.3	
8.4			
Mean 8.7		0.6	

Table 5 *Sorptive powers of chitin powder before and after grinding*

(A portion of chitin was taken from the original sample and ground in a mechanically operated agate mortar. Portions (0.1 g) of the two samples of chitin were agitated with 20 ml samples of 0.05% DDT suspension in 5% ethanol in water for 3 hr, and the amount of DDT sorbed by the chitin was estimated.)

DDT retained by chitin (mg/g, corrected for blanks)			
Before grinding		After grinding	
16	18	43	47
22	22	41	52
19	21	48	53
Mean 19 ± 3	20 ± 2	44 ± 4	51 ± 3

Table 6 *Action of various substances on colloidal suspension of DDT*

(Weighed portions of the substances under trial were agitated with 20 ml portions of 0.05% DDT colloidal suspension in 5% ethanol in water. The amount of DDT retained was estimated immediately after addition of the material and at some later time. Blanks were also run to determine the amounts of DDT retained by the filters.)

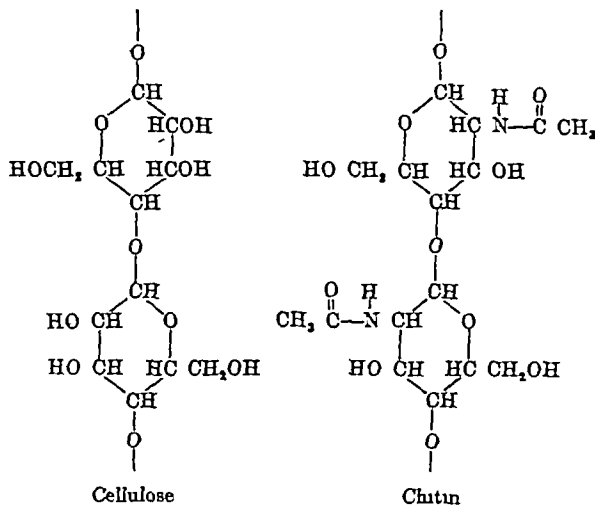
DDT retained		(mg, corrected for blank)	
At zero time		After 2 hr	Blank
Gooch asbestos, 0 10 g portions			
6 0		1 8	0 8
1 1		-0 1	0 8
5 2		6 4	0 7
6 4		1 0	0 3
5 5		3 4	1 9
2 8		0 7	1 4
Sheep's wool, 0 20 g portions			
-0 5		0 5	1 1
0 0		1 1	0 8
-0 4		1 4	—
0 0		0 9	0 9
0 0		1 2	0 8
0 0		0 6	0 8
Cellulose, (1) Fibres of cotton wool, 0 10 g portions			
0 8		0 9	0 9
0 9		0 8	0 8
1 2		—	—
0 20 g portions			
1 5		1 1	0 7
1 5		1 0	1 1
1 8		1 3	1 1
Cellulose, (2) Fibres from pulped filter paper, 0 10 g portions			
7 8		7 7	0 6
8 3		8 8	0 5
8 3		8 6	0 6

	After 0.5 hr	After 1.0 hr	After 1.5 hr	Blank
3.4	2.1	1.9	3.0	1.6
6.4	3.9	2.4	4.4	1.4

DISCUSSION

Since the agitation of sand with a colloidal suspension of DDT does not cause any significant increase in the amount of DDT retained by the filter itself from an untreated suspension (Table 1), it is clear that chitin must sorb DDT from its suspension, and does not cause the disperse phase to separate merely by mechanical action. The DDT retained by asbestos and filter paper fibres (Table 6) appears to be held mechanically, since the amount retained is apparently independent of time. If the action were other than mechanical, cellulose in other forms than filter paper pulp would be expected to retain DDT from colloidal suspensions, but cellulose in the form of cotton wool, in which the fibres are longer, and thus do not form such a compact pad, does not retain DDT. It therefore appears that DDT is not sorbed by cellulose. A small amount of DDT is retained by sheep's wool (Table 6), but much less than that sorbed by chitin. This rather weak affinity of wool for DDT is in line with the results reported by West & Campbell (1946).

Since the structural configuration of chitin and cellulose are very similar (Clark & Smith, 1936), it may be concluded that sorption occurs by a physico-chemical or chemisorption process associated with the presence of amino groups in the sorbing substance.



The relative lack of sorptive capacity of wool as compared with chitin may possibly be related to molecular configuration rather than to differences in surface area of equal weights of the two substances.

The increase in sorptive power of chitin as a result of grinding (Table 5) indicates that sorption is a surface phenomenon. Unfortunately, owing to the irregular shapes of the laminar particles, it was not possible to estimate the surface area of the chitin.

powders, and thus to relate quantitatively surface area and the amount of DDT sorbed

Comparison of the two sets of results in Tables 2 and 4 show that the rate at which the initial rapid sorption takes place depends on the concentration of the suspension used. This is further borne out by the results set out in Table 3, which records the amount of sorption by a fixed amount of chitin in a fixed time from colloidal suspensions of differing concentrations.

Consideration of the amount of DDT sorbed by a given mass of chitin (Tables 2 and 4) suggests that sorption takes place rapidly at first, and then either proceeds much more slowly or ceases altogether. Although the rate of sorption of DDT by chitin is partly dependent on the concentration of the suspension used, the apparent cessation of sorption (Tables 2 and 4) can be explained on the basis of the chitin becoming saturated with DDT, which again is the only possible explanation for the apparently constant amount of DDT sorbed by a unit weight of chitin in some experiments (Tables 1 and 2).

Although the molecular amounts of DDT analogues sorbed by a given weight of chitin from equimolecular suspensions (Table 4) are not significantly

different, there appears to be a tendency for the less toxic analogues to be sorbed to a greater extent than the more toxic analogues. The rates at which the various analogues are sorbed appear to be approximately equal, the differences being well within experimental error.

SUMMARY

1 DDT and its analogues are readily sorbed by chitin from colloidal suspension. The sorptive power appears to be peculiar to chitin since cellulose, wool (protein fibre) and silica powder do not possess this power.

2 The rate of sorption depends on the concentration of the colloidal suspension.

3 The total amount sorbed by a given weight of chitin appears to depend on the surface area of the chitin.

4 The molecular amounts of DDT and its analogues sorbed from suspensions of equimolecular concentrations are approximately equal, as are apparently the rates of sorption.

5 There is no significant correlation between the sorption of DDT analogues on chitin and their toxicity.

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Studies on Biological Methylation

11 THE ACTION OF MOULDS ON INORGANIC AND ORGANIC COMPOUNDS OF ARSENIC

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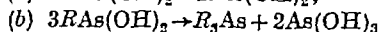
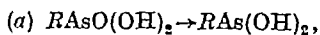
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Methylation by moulds: general and selective

The conversion of arsenites, selenites and tellurites to trimethylarsine, dimethyl selenide and dimethyl telluride in cultures of *Scopulariopsis brevicaulis* has been described by Challenger & Higginbottom (1935) and in Parts I-VII and IX of this series (for references see Challenger, 1945). The theoretical aspects of the subject are discussed in Part VIII (Challenger, 1942, 1945). This methylating action is also exerted on salts of alkyl- and dialkyl arsonic acids, $RAAsO(OH)_2$ and $R_2AsO(OH)$ (Challenger, Higginbottom & Ellis, 1933; Challenger & Ellis, 1935; Challenger & Rawlings, 1936) giving $RAAs(CH_3)_2$ and

R_2AsCH_3 , and on salts of the alkylseleninic acids, $RSeO_2H$ (Bird & Challenger, 1942) giving $RSeCH_3$. Thom & Raper (1932) reported that several other moulds volatilized inorganic arsenic, doubtless as trimethylarsine, although they did not examine the product. Some of these organisms have been studied by Bird & Challenger (1939, 1942) with similar results. *Penicillium chrysogenum*, *P. notatum* and *P. notatum* sp. produce dimethyl telluride from potassium tellurite and dimethyl selenide from both sodium selenite and methaneseleninic acid. The three last named moulds are, however, incapable of producing trimethylarsine from arsenious acid under the usual conditions. This recalls the statement of

Ségale (1903) who had obtained a mould (unspecified) which produced a volatile unidentified selenium compound, but was inert towards arsenic. No other workers have recorded any examples of this selective methylation. Further instances are now presented, and the interesting fact has emerged that *P. chrysogenum*, *P. notatum*, *P. notatum* sp and two strains of *Aspergillus niger* exert a selective action on compounds of arsenic and readily methylate sodium methylarsonate, $\text{CH}_3\text{AsO}(\text{ONa})_2$, and in most cases sodium cacodylate, $(\text{CH}_3)_2\text{AsOONa}$ to trimethylarsine, but are inert to arsenious acid. It could, however, be suggested that this conversion is not necessarily a methylation, but might involve a reduction followed by a disproportionation



Thus, Grisebawitsch Trochimovskii (1928) states that the oxide $\text{C}_6\text{H}_5\text{AsO}$ which results from the action of alkali on ethyldichloroarsine $\text{C}_2\text{H}_5\text{AsCl}_2$, is converted to triethylarsine and sodium arsenite. This possibility is, however, excluded by the behaviour of sodium ethylarsonate $\text{C}_2\text{H}_5\text{AsO}(\text{ONa})_2$ and sodium allylarsonate $\text{CH}_2=\text{CHCH}_2\text{AsO}(\text{ONa})_2$ in bread cultures of *A. niger* and of *Penicillium chrysogenum* respectively, dimethylethylarsine and dimethylallylarsine being produced in a pure condition. If reduction and disproportionation only had occurred, the products would have been triethylarsine and triallylarsine respectively. Had methylation of any arsenous oxide, simultaneously produced (see equation (b)), taken place, trimethylarsine would have been formed in addition. Since only dimethyl ethylarsine and dimethylallylarsine are evolved from the cultures, the reaction with these three moulds is therefore a methylation, as in the case of the analogous experiments with *Scopulariopsis brevicaulis*.

Three of the moulds under discussion, *Penicillium chrysogenum*, *P. notatum* and *P. notatum* sp exert no such selective action on selenium compounds, but convert sodium selenite and methane ethane and propane 1 selenenic acids to dimethyl, methylethyl and methyl n propyl selenides respectively (Bird & Challenger, 1942). The inability of the five organisms mentioned above to methylate inorganic arsenic must be associated with the earliest stages of the methylation process, which, according to any theory of biological methylation, involves the formation of methylarsonic acid $\text{CH}_3\text{AsO}(\text{OH})_2$. The green *Penicillia* and *Aspergillus niger* readily convert this compound to trimethylarsine. The selective action may be connected with the electron releasing effect of the alkyl groups, which would render electrons more readily available on the arsenic atom and the oxygen atom to which it is attached thus facilitating both reduction and attachment of a methyl group.

Aromatic arsonic acids and Scopulariopsis brevicaulis

Phenylarsonic acid yields no volatile arsine in cultures of *S. brevicaulis* (Challenger & Rawlings, 1936, 1937). It seemed possible that 2,4 xylylarsonic acid containing two electron releasing methyl groups might be more reactive, but in bread cultures of *S. brevicaulis* no xylyldimethylarsine or other volatile arsine was produced.

We have now found that phenyldimethylarsine is not evolved from cultures containing phenyl arsonoxide ($\text{C}_6\text{H}_5\text{AsO}$) or phenylmethylarsonic acid ($\text{C}_6\text{H}_5\text{As}(\text{CH}_3)\text{OOH}$). An explanation based on the difficult volatility of phenylmethylarsines would appear to be excluded, because it has now been shown that the nitrate of phenyldimethylarsine oxide ($\text{C}_6\text{H}_5\text{As}(\text{CH}_3)_2\text{O}$) is readily reduced by bread cultures of *S. brevicaulis* giving phenyldimethylarsine ($\text{C}_6\text{H}_5\text{As}(\text{CH}_3)_2$), which can be removed in sterile air and characterized as the mercurichloride. Were the mould able to methylate phenylarsonic acid to phenyldimethylarsine the oxide $\text{C}_6\text{H}_5\text{As}(\text{CH}_3)_2\text{O}$ would be an intermediate compound, on the basis of either the transmethylation or the formaldehyde theory of biological methylation (Challenger, 1942, 1945).

This easy reduction of phenyldimethylarsine oxide nitrate (phenyldimethylhydroxyarsonium nitrate) recalls the formation of trimethyl- and tri n propyl arsines from trimethylhydroxyarsonium nitrate and tri n propylarsine oxide respectively (Challenger & Higginbottom, 1935), of diethyl sulphide from diethyl sulphoxide, and of dimethyl selenide from dimethylhydroxyselenonium nitrate (Challenger & North, 1934), in cultures of *S. brevicaulis*. On the other hand, trimethylamine oxide hydrate and trimethylhydroxyammonium chloride give no trimethylamine in bread cultures of this mould. In view of the enzymic formation of trimethylamine from its oxide in fish this negative result is interesting.

Attempt to test the formaldehyde hypothesis of biological methylation

This hypothesis assumes the formation of hydroxymethylarsonic acid ($\text{HOCH}_2\text{AsO}(\text{OH})_2$) from arsenious oxide and its subsequent reduction to $\text{CH}_3\text{AsO}(\text{OH})_2$. This acid has not been synthesized and a crucial test of the hypothesis has not been possible (Challenger *et al.* 1933, Challenger & Higginbottom, 1935). It appeared possible that the behaviour of the condensation product of phenylarsine ($\text{C}_6\text{H}_5\text{AsH}_2$) and acetaldehyde ($\text{C}_6\text{H}_5\text{As}(\text{CHOHCH}_3)_2$) (Palmer & Adams, 1922) in cultures of *S. brevicaulis* might throw some light on the problem, since this should yield phenyldimethylarsine. In glucose Czapek Dox cultures of the mould, however, the compound underwent hydrolysis yielding acetalde-

hyde, its instability recalling that of the condensation product of ethylthiol and acetaldehyde (Levi, 1932, Challenger & Rawlings, 1937) The behaviour of this hydroxyarsine to mercuric chloride was studied in view of its possible volatilization from the cultures and reaction with this reagent in the absorption train With excess of a saturated aqueous solution the arsine gave a white precipitate, which, on boiling with ethanol, gave mercury, mercurous chloride, phenylmercury chloride, arsenobenzene, acetaldehyde, and a trace of a phenylhalogenarsine, probably phenyldichloroarsine

It is well known that choline and betaine can engage in transmethylation in rats (Simmonds, Cohn, Chandler & du Vigneaud, 1943, du Vigneaud, Simmonds, Chandler & Cohn, 1946), and mycological methylation may proceed by a similar process (Challenger, 1945) No appreciable effect on trimethylarsine production was observed by Challenger & Higginbottom (1935) on addition of choline or betaine to bread cultures of *S. brevicaulis* containing arsenious oxide The effect of 'triethylcholine' chloride $[(C_2H_5)_3NCH_2CH_2OH]^+Cl^-$ and 'triethylbetaine' $^+(C_2H_5)_3NCH_2COO^-$ in similar cultures has now been investigated Careful examination of the dimercurichloride so obtained failed to show the presence of an ethyl derivative This recalls the work of du Vigneaud (1941), who showed that when ethionine (*S* ethylhomocysteine) and choline are fed to rats on a methionine free diet no growth resulted, indicating that de ethylation of ethionine to homocysteine does not occur Results of the present and earlier papers are summarized in Table 1

EXPERIMENTAL

Behaviour of different moulds in bread cultures to various sources of arsenic

Unless otherwise stated the moulds were obtained from the National Collection of Type Cultures at the Lister Institute, London (NCTC)

Arsenious oxide The following moulds were found to convert As_2O_3 to trimethylarsine when the oxide was present to the extent of about 0.2% in the bread *Aspergillus glaucus* (Birkbeck) (NCTC no 1216) and *A. versicolor* (NCTC no 1883) The quantity produced was, however, much smaller than with the organisms and substrates described below The trimethylarsine was absorbed in acid $HgCl_2$ (Bignelli's solution) (Challenger *et al* 1933), and characterized as the dimercurichloride, the melting point (decomp) of the unrecrystallized product varied from 255 to 260° Satisfactory mixed melting points were carried out in these and most of the following experiments Trimethylarsine dimercurichloride has been repeatedly examined during the course of these investigations (Challenger *et al* 1933, Challenger & Higginbottom, 1935) We find that, using bread cultures of *Scopulariopsis brevicaulis* (Sacc) Baier (*Baarn List of Fungi*, 1932) containing about 0.1% of As_2O_3 , roughly 70% of the added arsenic may be recovered as trimethylarsine dimercurichloride during

Table 1 Behaviour of different moulds to various sources of arsenic, selenium and tellurium in bread cultures

Mould	NCTC no	As_2O_3	Na_2HAsO_4	Na_2SeO_3	K_2TeO_3	Compound added to culture				
						Identified product				
						$CH_3AsO(ONa)_2$	$(CH_3)_2AsO(ONa)_2$	$C_2H_5AsO(ONa)_2$	$C_2H_5As(CH_3)_2$	$C_2H_5As(CH_3)_2$
<i>S. brevicaulis</i>	1362, 580	$(CH_3)_2As$	$(CH_3)_2As$	$(CH_3)_2Se$	$(CH_3)_2Te$	$(CH_3)_2As$	$(CH_3)_2As$	$C_2H_5As(CH_3)_2$	$C_2H_5As(CH_3)_2$	$C_2H_5As(CH_3)_2$
<i>P. notatum</i>	4222	None	—	$(CH_3)_2Se$	Doubtful $(CH_3)_2Te$, odour only	$(CH_3)_2As$	$(CH_3)_2As$	—	—	—
<i>P. chrysogenum</i>	580	None	—	$(CH_3)_2Se$	$(CH_3)_2Te$	$(CH_3)_2As$	$(CH_3)_2As$	—	—	—
<i>P. notatum</i> sp	—	None	—	$(CH_3)_2Se$	$(CH_3)_2Te$	$(CH_3)_2As$	$(CH_3)_2As$	—	—	—
<i>A. niger</i>	—	None	—	Na_2SeO_4	—	$(CH_3)_2As$	$(CH_3)_2As$	—	—	—
<i>A. fischeri</i>	3779	None	(No odour, no redn)	(No odour, no redn)	(No odour, no redn)	$(CH_3)_2As$	$(CH_3)_2As$	—	—	—
<i>A. niger</i> 17	—	—	—	—	—	$(CH_3)_2As$	$(CH_3)_2As$	—	—	—
<i>A. glaucus</i>	1216	$(CH_3)_2As$	(No odour, no redn)	(No odour, no redn)	(No odour, no redn)	$(CH_3)_2As$	$(CH_3)_2As$	—	—	—
(Birkbeck)	—	—	—	—	—	$(CH_3)_2As$	$(CH_3)_2As$	—	—	—
<i>A. versicolor</i>	1883	$(CH_3)_2As$	(No odour, no redn)	(No odour, no redn)	(No odour)	$(CH_3)_2As$	$(CH_3)_2As$	—	—	—

24 months' aspiration *S. brevicaulis* (strain Derr N C T C no 1362) yielded about 80% in 10 months. An attempt was made by Dr C Simons in these laboratories to increase the rate of trimethylarsine formation by this mould by cultivating the strain *S. brevicaulis* (Sacc) Baurer (*Baarn List of Fungi*, 1932) for 33 generations on a potato agar medium containing 0.12–0.15% of As_2O_3 . No appreciable alteration in the rate of methylation could be detected, when the 'trained' mould was inoculated on to sterile bread containing 0.1% of As_2O_3 .

Sodium methylarsenate ($CH_3AsO(ONa)_2$) The following moulds gave trimethylarsine, characterized as the dimer curichloride (unrecryst), the melting point (decomp) varying from 252 to 263°. *Penicillium chrysogenum* (N C T C no 589), *P. notatum* (N C T C no 4222), *P. notatum* sp (see Burd & Challenger, 1933), *Aspergillus niger* and 'A niger 17' (obtained from Dr T K Walker of the Biochemistry Department, The College of Technology, Manchester), *A. fischeri* (N C T C no 3779), *A. glaucus* (Birkbeck) (N C T C no 1216) and *A. versicolor* (N C T C no 1883). The concentration of the sodium methylarsenate in the bread was 0.2–0.25% except in the case of *A. niger*, where it was 0.07%. In the case of *Penicillium notatum* and *Aspergillus niger* 17 the trimethylarsine was also characterized as trimethyl hydroxyarsonium nitrate ($(CH_3)_3As(OH)NO_3$) (Challenger *et al* 1933), m p and mixed m p 124 and 126° respectively.

Sodium cacodylate ($(CH_3)_2AsOONa$) Trimethylarsine was again obtained and characterized as before in cultures of the following *Penicillium chrysogenum*, *P. notatum*, *Aspergillus niger* and 'A niger 17'. The usual concentration was 0.2 and 0.25% in the bread. *Penicillium notatum* sp gave only a trace of the dimer curichloride.

Ethylarsonic acid ($C_2H_5AsO(OH)_2$) at a concentration of 0.13% in the bread gave with *Aspergillus niger* dimethyl ethylarsine, characterized as the mercurichloride, m p and mixed m p 238° (Challenger *et al* 1933).

Sodium allylarsenate ($CH_2CHCH_2AsO(ONa)_2$), concentration 0.2%, gave with *Penicillium chrysogenum* dimethyl allylarsine, characterized as the mercurichloride, m p and mixed m p 209°. Here again no reduction of the double linkage in the allyl group occurs (Challenger & Ellis, 1935).

Ethyl n-propylarsonic acid ($C_2H_5(C_3H_7)_2AsOOH$), concentration 0.17% in the bread cultures, gave with 'Aspergillus niger 17' methylethyl n-propylarsine, characterized as the dimer curichloride, m p and mixed m p 171–172° decomp between 180 and 192° (Challenger & Rawlings, 1936).

n-Propylarsonic acid ($C_3H_7AsO(OH)_2$), concentration 0.34% in the bread cultures, gave with *Penicillium notatum* dimethyl n-propylarsine dimer curichloride, m p and mixed m p 195–197°, decomp 199–200° (Challenger & Ellis 1935).

The non-occurrence of methylation of arsenious oxide with certain moulds

In preliminary experiments the following organisms appeared unable to convert As_2O_3 to trimethylarsine in bread cultures *P. chrysogenum*, *P. notatum*, *P. notatum* sp, *Aspergillus niger*, *A. niger* 17' ($As_2O_3=0.02\%$) and *A. fischeri* ($As_2O_3=0.2\%$). Nevertheless, it was decided to treat five of these organisms with varying concentrations of As_2O_3 . Six cultures of each mould on 50 g of bread without added water were incubated for 9 days, and a sterile aqueous solution of As_2O_3 (15 ml 20 ml in F) added to each group of five flasks (groups A–F) to give concentrations in the bread of 0.02, 0.05, 0.10, 0.20, 0.30 and

0.40%. During the first 3–4 days the *Penicillium notatum* flasks D, E and F had a very faint odour, barely detectable on the 10th day. On the 14th day an odour was detected in flask C, though with difficulty. With *P. chrysogenum* a very faint odour appeared to be present in E and F. The two strains of *Aspergillus niger* gave no odour, nor did *Penicillium notatum* sp, until after about 2 months, by which time slight contamination may have occurred.

Appearance of the cultures With the six cultures of *P. notatum* the bread was covered with mycelium about 3 days after addition of As_2O_3 . After 9 weeks the cultures showed almost black patches, especially flasks D, E and F. Subcultures from these on to wort agar failed to grow during 45 days, although they were re-inoculated on the 5th day. All the cultures of *P. chrysogenum* and *P. notatum* sp grew well. The strains of *Aspergillus niger* grew well in groups A and B, but in D, E and F very little growth occurred after addition of the As_2O_3 .

Penicillium notatum and arsenious acid It was decided to test for trimethylarsine evolved by *P. notatum* by chemical means. Five 1 l flasks, each containing 150 g of bread, were sterilized, inoculated with spores of *P. notatum* and incubated for 7 days at room temperature. To each were added 45 ml of sterile 1% (w/v) As_2O_3 solution (conc in bread 0.3%), and aspiration commenced through two tubes of Bignelli's solution. No deposit appeared and no odour was detected during 30 days. The cultures in two flasks were then quite white, the green sporing area being completely covered. Subcultures were made from these two flasks, each to two wort-agar slopes, to see if this white growth was contamination or sterile mycelium due to the As_2O_3 . After 5 days' incubation at room temperature all four slopes showed only typical green colonies of *P. notatum*. One plate culture was also made from each flask with the same result. *P. notatum* would therefore appear not to produce trimethylarsine from As_2O_3 , in agreement with the original observation.

Growth of P. notatum on a liquid medium containing arsenious oxide from the outset Seven 250 ml conical flasks, each containing 100 ml of 5% (w/v) glucose Czapek Dox solution with concentrations of As_2O_3 of 0.01, 0.02, 0.04, 0.08, 0.16, 0.32 and 0.64%, were sterilized, inoculated and incubated at room temperature. After 10 days all the flasks except the last showed a white surface growth which was compact, raised and abnormal. During another 4 days, growth continued in concentrations 0.01–0.16%, increase was slow with 0.32%.

Arsenic acids containing the phenyl group and Scopulariopsis brevicaulis

The strain used was designated *S. brevicaulis* (Sacc) Baurer in the *Baarn List of Fungi* (1932), and was distinguished in the Leeds laboratories as strain A (Challenger *et al* 1933). It was grown on 150 g of bread in 1 l flasks. Solutions containing the arsenic acids were made up thus: (a) Phenylarsonic acid (1.2 g) carefully purified by three crystallizations from water, m p 161–162°, was dissolved in water (100 ml) containing $NaHCO_3$ (1.1 g) and sterilized; (b) 2,4-Xylylarsonic acid (0.6 g) (Michaelis, 1902) was dissolved in sterile water (80 ml) containing $NaHCO_3$ (0.65 g); (c) Benzylarsonic acid (0.75 g), three times recrystallized from water without change in m p 196–198° after air drying (the acid readily loses water), was suspended in sterile water (80 ml).

25 ml of solutions (a) and (b) and of suspension (c) were added to bread cultures of the mould (three in each case), and volatile products aspirated through acid HgCl_2 solution. A precipitate formed after some weeks in each case, but was shown to be free from the mercurichloride of a tertiary arsine $\text{RAs}(\text{CH}_3)_2$ and to consist of the ammonia compound of HgCl_2 , which was prepared for comparison.

Behaviour of arsenious oxide in bread cultures of Scopulariopsis brevicaulis containing 'triethylcholine' chloride and the triethylbetaine of aminoacetic acid

'Triethylcholine' chloride (25 ml of a sterilized 8% (w/v) aqueous solution) was added to each of three flasks of sterile bread, which were then inoculated with the mould and incubated for 4 days at 32° and 4 days at room temperature. Each flask then received 25 ml of a sterile aqueous 1.2% (w/v) solution of As_2O_3 . Aspiration through Bignelli's solution gave during 40 days only 0.22 g of trimethylarsine dimercurichloride, m.p. 266–267° decomp. A mixed melting point confirmed its identity 'Triethylbetaine' (25 ml of a sterilized 3% (w/v) aqueous solution) was added to each of four 1 l. flasks containing 150 g of sterile bread, which were then inoculated and incubated for 4 days at 32° and 2 days at room temperature. Addition of 25 ml of sterilized aqueous 1.2% As_2O_3 solution gave as before during 28 days 0.2 g trimethylarsine dimercurichloride, m.p. 266–267° with sintering from 263°. The mixed melting point with an authentic specimen of m.p. 267° was 266°. On the 40th day, a further deposit was removed, m.p. 265 and 266° after recrystallization from very dilute HgCl_2 solution. Similar results were obtained with two cultures containing 2% and one with 1% of added 'triethylbetaine' connected in series.

Scopulariopsis brevicaulis and aromatic compounds of arsenic

S. brevicaulis and phenylarsonic acid The acid (4 g) and Na_2CO_3 (2 g) were dissolved in water (150 ml). 25 ml of the solution were added to each of six 1 l. flasks containing 3% (w/v) glucose Czapek-Dox medium (250 ml) on which a good mycelium had formed. Sterile air was then drawn through the flasks in series with Bignelli's solution during 30 days, but no deposit formed, indicating the absence of phenyldimethylarsine. The whole of the medium was then filtered, concentrated to 400 ml and a portion tested for phenyldimethylarsine oxide with picric acid, but no precipitate formed. The main bulk was then extracted ten times with ether, which only removed traces of phenylarsonic acid. A portion of the concentrated medium gave with HCl , SO_2 and I_2 (see p. 82) a yellow oil, which with ethanolic HgCl_2 yielded phenylmercury chloride, m.p. and mixed m.p. 247° in admixture with a specimen obtained in a similar manner from authentic phenylarsonic acid (see p. 83). From the bulk of the concentrated medium 30% of the total phenylarsonic acid was recovered as the thorium salt (Rice, Fogg & James, 1926). Quantitative recovery was not expected due to the material having been used for preliminary tests. Removal of thorium as oxalate, and of oxalic acid with H_2O_2 yielded phenylarsonic acid. It sintered at 158°, in agreement with an authentic specimen.

S. brevicaulis and phenylarsenoxide Addition of 0.2% of the oxide to each of four cultures of the mould prepared as in Exp. 1 gave no odour during 6 months, and the insoluble

oxide appeared unchanged. Neither reduction to phenylarsine nor production of phenyldimethylarsine occurred.

S. brevicaulis and phenylmethylarsonic acid For the preparation of the acid see Burrows & Turner (1920, 1921), Gibson & Johnson (1928). Five 1 l. conical flasks, each containing a good growth of the mould on 5% (w/v) glucose Czapek-Dox solution (200 ml), were treated with 25 ml of sterile 2% aqueous sodium phenylmethylarsonate. There was no odour and no precipitate in aqueous HgCl_2 after aspiration for 5 weeks. Evaporation of the slightly acidified medium, extraction with ethanol and treatment with HCl , SO_2 and excess I_2 gave a yellow oil. This with ethanolic HgCl_2 yielded a white precipitate, which on recrystallization from ethanol sintered at 148°, but did not melt. Its behaviour was identical with that of the mercurichloride of authentic phenylmethylarsine (Found, for the specimen from the culture: C, 14.5, H, 1.8, halogen, 33.2; $\text{C}_6\text{H}_5(\text{CH}_2)_2\text{AsI}$ HgCl_2 requires C, 14.9, H, 1.42, halogen 35.0%). The medium therefore appeared to contain unchanged phenylmethylarsonic acid or phenylmethylarsenoxide.

S. brevicaulis and phenyldimethylhydroxyarsonium nitrate Six 1 l. flasks, each containing 150–200 g of bread slightly moistened with water, were inoculated with an aqueous spore suspension of the mould (strain *Saccharomyces* Bainier, see p. 80). After 4 days the odourless solution of the hydroxyarsonium nitrate in sterile water was added, the concentration in the bread being 0.17%. In a few hours an arsenical odour was apparent, and the flasks were connected with sterile bungs and delivery tubes as in earlier experiments (Challenger *et al.* 1933). A stream of sterile air was then aspirated through the cultures into saturated aqueous HgCl_2 . A deposit formed after 2 days, which when recrystallized from hot water had m.p. and mixed m.p. 196° with authentic phenyldimethylarsine mercurichloride, m.p. 196°. A second crop sintered from 185 to 195°. After one recrystallization the melting point rose to 196°. Later on, due to the production of NH_3 in the cultures, the precipitate increased rapidly. It was extracted with ethanol and then with boiling water leaving the ammonia compound of HgCl_2 . The phenyldimethylarsine mercurichloride obtained (0.62 g) represented 23% of the original hydroxyarsonium nitrate. In a control experiment six test tubes, each containing approximately 7 g of sterile bread and 0.17% of the hydroxyarsonium nitrate, gave no odour or any deposit in aqueous HgCl_2 during 28 days.

S. brevicaulis and phenylbis(1-hydroxyethyl)arsine The arsine (3 g) was suspended in sterile water (150 ml), and 25 ml added to each of six well-grown cultures on glucose Czapek-Dox solution (200 ml). Sterile air was drawn over the cultures into saturated HgCl_2 . After a few days the only odour recognizable was that of the original arsine. A slight precipitate in the absorption bottles was shown to be HgCl_2 . On placing aqueous 2.4 dinitrophenylhydrazine hydrochloride at the end of the absorption train a yellow solid formed. When recrystallized from ethanol this melted at 156° alone and in admixture with authentic acetaldehyde 2.4 dinitrophenylhydrazone of m.p. 156°.

Reactions of some aromatic compounds of arsenic

Conversion of phenylarsonic acid to phenylmercury chloride The acid was converted to phenyldiodoarsine. In acetone this gave a white addition product with aqueous HgCl_2 . Heating this with water gave a red solid and hot

acetone produced a red solution, doubtless due to HgI_2 . Crystallization from ethanol yielded white glistening plates, m.p. 242° . On successive recrystallizations from acetone, ethanol and acetone, the m.p. were 247 , 246 and 247° , not depressing the m.p. (251°) of phenylmercury chloride.

Phenyldiethylarsine mercurichloride The arsine (c. 1 g) (Burrows & Turner, 1920) was shaken with excess of saturated aqueous HgCl_2 until the mixture was odourless. The white precipitate was recrystallized from ethanol (m.p. 127 , 129 , 129°) (Found Cl, 16.2, Hg, 42.2. $\text{C}_{10}\text{H}_{13}\text{Cl}_2\text{AsHg}$ requires Cl, 14.74, Hg, 41.05%).

Phenylbis(1 hydroxyethyl)arsine and mercurichloride A concentrated ethanolic solution of the arsine (Palmer & Adams, 1922) was shaken with excess of saturated aqueous HgCl_2 for several hours. The white precipitate quickly became yellow. It still smelled slightly of the arsine and was washed with water and a little ethanol. It blackened and decomposed from 90° . Warming with ethanol gave Hg_2Cl_2 and mercury but no Hg_2O . The ethanolic filtrate (B) gave white crystals, m.p. 254° , an identical product also slowly separated from the aqueous filtrate (A) from the crude mercurichloride. It contained halogen and mercury but no arsenic, and was shown by mixed m.p. (255°) to be phenylmercury chloride (m.p. 252°). Concentration of the filtrate (B) gave a white crystalline material, m.p. 187 – 194° , which was twice recrystallized from benzene-ether, m.p. 212 and 213° . It contained arsenic but no halogen or mercury, and was unaffected by NaOH , NH_3 or HCl . It was readily attacked by HNO_3 , KMnO_4 , H_2O_2 and I_2 and was insoluble in water. (Found C, 48.3, H, 3.5, mol.wt (camphor), 401. Calc for $\text{C}_{12}\text{H}_{15}\text{As}_2$, C, 47.4, H, 3.3%, mol.wt 304.) Arseno benzene, m.p. 212° , gives high values in ebullioscopic molecular weight determinations in benzene (Michaelis & Schäfer, 1913). Its identity was confirmed (1) by oxidation with HNO_3 when the product (phenylarsonic acid) gave a white gelatinous thorium salt (see p. 82), and (2) by treatment with Cl_2 giving a yellow oil ($\text{C}_6\text{H}_5\text{AsCl}_4$) which soon crystallized ($\text{C}_6\text{H}_5\text{AsCl}_4$). A portion of the filtrate (B) with 2,4-dinitrophenylhydrazine hydrochloride gave a

copious yellow precipitate, m.p. and mixed m.p. 150° , with authentic acetaldehyde 2,4-dinitrophenylhydrazone, m.p. 150° . This was also obtained from the aqueous filtrate (A). Complete evaporation of (B) left only a few drops of a yellow lachrymatory oil containing chlorine. It was probably phenyldichloroarsine.

SUMMARY

1 *Scopulariopsis brevicaulis* converts arsenious acid and methylarsonic acid to trimethylarsine, other alkylarsonic acids yield alkyltrimethylarsines. *Penicillium notatum*, *P. notatum* sp., *P. chrysogenum* and two strains of *Aspergillus niger* are inert to arsenious acid, but can methylate alkylarsonic acids. *A. glaucus* gives trimethylarsine with arsenious and methylarsonic acids. All cultures were on bread.

2 Phenylarsonic and phenylmethylarsonic acids in glucose Czapek-Dox medium, and *m*-xylylarsonic and benzylarsonic acids in bread cultures give no volatile arsenic compounds with *Scopulariopsis brevicaulis*. Phenyldimethylhydroxyarsonium nitrate, however, is readily reduced in bread cultures to phenyldimethylarsine which readily volatilizes.

3 In cultures of *S. brevicaulis* on glucose Czapek-Dox medium phenylbis(1-hydroxyethyl)arsine does not give phenyldiethylarsine, but is hydrolyzed to acetaldehyde. The behaviour of the hydroxyarsine to mercuric chloride has been studied.

4 In bread cultures of *S. brevicaulis* containing arsenious acid and 'trimethylcholine' chloride or 'triethylbetaine' only trimethylarsine is evolved, trans-ethylation apparently does not occur.

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The Chemical and Immunological Properties of Phosphorylated Proteins

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The natural phosphoproteins such as casein and ovovitellin offer much of interest, chemically and immunologically, and many investigators have studied both these and artificially phosphorylated proteins, to determine how the phosphate groups are linked to the protein molecule, comparisons of the chemical properties of both types of protein have also been made by several workers. Phosphoryl chloride was used as phosphorylating agent by Bechhold (1901), Neuberg & Pollak (1910) and Neuberg & Oertel (1914). These methods were modified by Rimington (1927), who treated the ice-cold protein solution with phosphoryl chloride in carbon tetrachloride, adding alkali at intervals to keep the solution faintly alkaline. Rimington described the preparation and properties of phosphorylated proteins prepared from casein, denatured serum globulin and dephosphorized casein.

A comprehensive chemical and immunological investigation of phosphorylated egg albumin was made by Heidelberger, Davis & Treffers (1941). They found that phosphorylation, which was accompanied by some denaturation, effected a profound change in the serological reactions of the egg albumin. Immunological investigations have also been made on phosphorylated casein (Ecker & Simon, 1928) and casein, dephosphorized casein and rephosphorized casein (Kesztyus & Kocsis, 1942).

Our own investigations with phosphorylated proteins were started with two main objects. It was hoped that the introduction into the protein molecule of phosphate groups containing radioactive P would yield labelled antigens which could be used for serological investigations, e.g. for studies on the fate of injected protein antigens and for quantitative studies on the precipitin reaction. It was also intended to study the nature of the linkage between the phosphate group and the protein in phosphoproteins and phosphorylated proteins, by immunochemical and isotopic tracer methods. Some of the results of these investigations are given in the present paper.

EXPERIMENTAL

Phosphorylation and dialysis The method used was essentially that described by Rimington (1927). Freshly distilled POCl_3 , dissolved in about 8 vol. of carbon tetra-

chloride, was added slowly to the protein solution cooled in ice water, varying amounts of POCl_3 were used, usually ranging from 0.1 to 0.5 g/g of protein. The mixture was stirred continuously and maintained at an alkaline pH (pink to phenolphthalein added as an internal indicator) by the addition of N or 4N NaOH at intervals during the addition of the POCl_3 and for some time afterwards. When no more NaOH was required, the aqueous layer was dialyzed at about 5° in cellophane sacs against 0.9% (w/v) NaCl (to which a little toluene had been added) until free from inorganic phosphate. In some of the earlier experiments distilled water was used as the dialyzing fluid. To determine the extent of the phosphorylation, total P and total N determinations were made on the original solution and on samples of the phosphorylated proteins precipitated from the solution in various ways. The results are usually expressed as a ratio (wt of P/wt of N).

Precipitation of the phosphorylated proteins In most of the experiments the phosphorylated protein was precipitated from solution by adjusting the pH to 4–4.6 with 0.1N HCl , using external indicators, the best results were obtained by using 2% (v/v) acetic acid for final adjustment of the pH to give maximal precipitation. Precipitation was least satisfactory and sometimes impossible with phosphorylated proteins of low P/N ratios. In several experiments the proteins were precipitated by the addition of 4 vol. of a 10% (w/v) solution of trichloroacetic acid (subsequently referred to as TCA). When these precipitates were washed with ethanol or acetone to remove TCA, some protein dissolved, but this was precipitated again by the addition of N NaOH and a few drops of conc. MgCl_2 solution. The unwashed precipitates obtained with TCA, when dried over CaCl_2 , had a horny texture. In other experiments the phosphorylated protein was precipitated with 6 vol. of ethanol, since the precipitates obtained with TCA gave lower P/N ratios than did those obtained with HCl , it was thought possible that some of the less firmly attached phosphate groups had been removed from the protein by treatment with TCA. The P/N ratios of ethanol precipitated phosphorylated proteins were almost always the same as those for the HCl precipitated product from the same solution, where this solution was free from inorganic phosphate. Where, however, the solution contained inorganic phosphate, the ethanol precipitated proteins gave a relatively high P/N ratio, and tests made with ^{32}P containing inorganic phosphate showed that this was due to the precipitation of appreciable amounts of inorganic phosphate by the ethanol. The results obtained with ethanol precipitated products have therefore been discarded, except where they refer to precipitates obtained from solutions free from inorganic phosphate.

P determinations These were made by King's (1932) modification of the Fiske & Subbarow (1925) method.

Preparation of phosphorylated proteins for immunization

'High P' proteins Sterile human serum (110 ml) was phosphorylated in two equal batches, using a POCl_3 /protein ratio of 0.5/1. After dialysis, the phosphorylated proteins were precipitated by acid (\approx HCl and 2% acetic acid) and redissolved in sterile 0.9% NaCl with the addition of a little \approx NaOH. Phenol (final concentration 0.25%) was also added. This solution had a protein content of 5.1% (w/v) and a P/N ratio of 0.14.

'Low P' proteins Sterile human serum (105 ml) was phosphorylated, dialyzed, precipitated and redissolved as above. The final solution contained 7.25% protein (P/N ratio 0.14) and 0.25% phenol. A mixture of this solution and 0.25 vol of 0.75M NaHCO_3 , H_2CO_3 buffer (pH 7.5) was placed in a stoppered bottle, the air in the bottle displaced by CO_2 and the bottle sealed and kept at 39° for 139 hr. The solution was then dialyzed against cold 0.9% NaCl to remove any free phosphate which had been split off at 39°, and 0.25% of phenol added. This solution contained 5.2% protein with a P/N ratio of 0.02.

Preparation of antigens for precipitin tests Rabbit plasma and chicken serum were phosphorylated using a POCl_3 /protein ratio of 0.5/1. The phosphorylated rabbit plasma had a P/N ratio of 0.17, some of this solution was kept at 37° with an equal volume of 0.75M NaHCO_3 , H_2CO_3 buffer (pH 7.5) for 4 days, followed by dialysis ('low P' rabbit proteins). In an attempt to obtain a phosphorylated antigen having a higher P/N ratio, rabbit serum was phosphorylated using a POCl_3 /protein ratio of 2.5/1, but the product had the same P/N value (0.17) as the phosphorylated rabbit plasma preparation described above. Gelatin solutions (0.25 and 0.5%) were treated with different amounts of POCl_3 (POCl_3 /protein ratios of 0.5 and 2.5/1 respectively). The amount of protein present after dialysis was, however, very small, and in both cases there was insufficient material for accurate P determinations.

Immunization The 'high P' and 'low P' phosphorylated human serum proteins were each injected into a group of five rabbits. Two rabbits of each group received an initial intravenous injection of 4.5 or 5 ml. of the solution, followed by five or six intraperitoneal injections of 5 ml. (rising gradually to 10 ml.) at intervals of 7-10 days. Sera from these rabbits were tested after the third and subsequent injections. The remaining three rabbits of each group received three courses of intraperitoneal injections each course consisted of four or five injections (each of 1.8 or 2.0 ml. of 5% protein solution) at intervals of 2-4 days, with an interval of 8-12 days between the courses. Sera for precipitin tests were collected after the second and third courses.

Precipitin tests The tests were made as described previously (Hopkins & Wormald, 1933). The concentration of antigen in the Tables refers to dilution (with 0.9% NaCl) of a solution of the antigen containing approx. 5% protein.

RESULTS

Phosphorylation of 'dephosphorized casein', serum globulin and serum albumin

'Dephosphorized casein' was prepared from 'soluble casein' (British Drug Houses Ltd) by the action of 0.25N NaOH (Plummer & Bayliss, 1906; Rimington & Kay, 1926), the

product being purified by two precipitations. The globulin and albumin were prepared from human serum by half and full saturation with $(\text{NH}_4)_2\text{SO}_4$, they were purified by repeated precipitation, and were finally dialyzed against 0.9% NaCl until free from $(\text{NH}_4)_2\text{SO}_4$. Sufficient 0.9% NaCl was added to each solution to give a protein concentration of 4.8% (w/v).

Each solution (20.8 ml. of dephosphorylated casein, globulin or albumin) was phosphorylated with 0.5 g. of POCl_3 (i.e. a POCl_3 /protein ratio of 0.5/1), as described above. During the course of phosphorylation the globulin and dephosphorized casein solutions became gelatinous, and adequate stirring was difficult, 0.9% NaCl, equal to about 0.25 of the total volume of the solution, was therefore added before the phosphorylation had been completed. The albumin solution was similarly diluted before phosphorylation. After dialysis the phosphorylated proteins were repeatedly precipitated with HCl, the precipitates being washed with distilled water to which a few drops of acetic acid had been added. P/N ratios, determined on the precipitates obtained in the 4th, 5th and 6th precipitations are given in Table 1.

The results showed that, under these conditions, phosphorylation of serum albumin, serum globulin and dephosphorized casein with POCl_3 readily effects the introduction of one P atom for every 33-38 N atoms in the protein molecule, this represents a P content of 1.0-1.07% for the phosphorylated proteins, calculated on the basis of 16% N in the protein. It is interesting to note that approximately the same amount of P was introduced into three different types of protein. The results also show that after three precipitations of these phosphorylated proteins with HCl, further acid precipitation yielded products of approximately constant P content.

Table 1. P contents of 'rephosphorized' casein and phosphorylated human serum albumin and globulin

Precipitation	P/N (weight ratios)		
	Phosphorylated albumin	Phosphorylated globulin	Rephosphorized casein
4th	0.062	0.087	0.083
5th	0.059	0.070	0.082
6th	0.057	0.065	0.060

Conditions of preparation of phosphorylated human serum proteins

Effect of varying the POCl_3 /protein ratios and other experimental conditions Since it was our intention to introduce ^{32}P into proteins for immunological investigations several experiments have been carried out to determine the amount of P which enters the protein when it is treated with amounts of POCl_3 considerably less than those used by Rimington (1927) and other investigators. In a typical experiment, three samples, each of 14.3 ml. of pooled human serum and containing 1 g. of protein, were treated with 0.5, 0.3 and 0.1 g. of POCl_3 respectively. The P/N ratios of the dialyzed solutions and of the acid precipitated products from the 1st, 2nd and 3rd precipitations showed that with the smaller amounts of POCl_3 less P was introduced into the

Table 2 Comparison of P/N ratios of human serum proteins phosphorylated under varying conditions

Material	Protein content (%)	Ratio POCl ₃ /protein	P/N ratio			
			Dialyzed solution	HCl precipitate		
				1st	2nd	3rd
Human serum	7.25	0.5	0.104	0.111	0.100	0.101
		0.3	0.078	0.083	0.074	0.071
		0.1	0.030	0.033	0.031	0.033
Freeze dried human serum	7.0	0.3	0.075	—	—	—
	12.5	0.3	0.095	—	—	—

protein (Table 2). With POCl₃/protein ratios of 0.5/1, 0.3/1 and 0.1/1, the resulting phosphorylated proteins contained 1.62, 1.14 and 0.53% of P respectively, calculated on a 16% N content for the protein. This would correspond respectively to the introduction of about 52, 37 and 17 P atoms into each protein molecule, assuming a mean mol. wt. of 10⁵ for the serum proteins.

Phosphorylation of a concentrated solution of human serum proteins. It was thought possible that less POCl₃ would be lost by hydrolysis if the solution to be phosphorylated had a higher protein content. This point was tested using a concentrated solution of serum proteins and a relatively low POCl₃/protein ratio. Samples of freeze dried human serum (kindly presented by Dr R. R. Race) were dissolved in water to give 12.5 and 7% protein solutions. These solutions were phosphorylated with sufficient POCl₃ to give a POCl₃/protein ratio of 0.3/1 and then dialyzed. The results recorded in the lower part of Table 2 showed that a saving of POCl₃ could be effected by the use of the more concentrated protein solution, but this method was not used in later experiments since it gave a greater amount of insoluble material than did phosphorylation of normal serum.

Stability of the phosphoryl group attached to proteins

Preliminary investigations had suggested that loss of phosphoryl groups occurred when the phosphorylated proteins were kept at 37°, even at or near neutrality, and the following experiments were therefore carried out to determine to what extent

this loss might occur under physiological conditions, e.g. after injection into the animal body.

Loss of P from phosphorylated human serum proteins, 'rephosphorized' casein and casein at 37°. Solutions of phosphorylated human serum proteins, casein, and in one case rephosphorized casein (all phosphorylated with a POCl₃/protein ratio of 0.5/1 and precipitated by HCl), were treated with equal volumes of either 0.75M NaHCO₃-H₂CO₃ buffer (pH 7.5) or veronal buffer (pH 6.8) and the mixtures sealed in bottles with a little toluene, and kept at 37°. Samples were taken at intervals and the proteins precipitated by TCA. The results (Table 3) showed that the phosphorylated human serum proteins had lost appreciable amounts of combined P when the solutions (at pH 6.8 or 7.5) were kept at 37°, at least one fifth of the combined P in 24 hr. and well over half in 6 days. Somewhat similar results were obtained in an experiment with rephosphorized casein (at pH 6.8). The P of casein is, however, much less labile under these conditions, and the loss in 6 days was usually less than 10%. In another experiment the incubation at pH 7.5 was continued for 20 days, with results similar to those recorded in Table 3, analysis of the TCA precipitated proteins showed that the phosphorylated serum proteins had lost about half their combined P during the incubation, whereas casein had lost little or none.

Table 3 P content of phosphorylated proteins kept at 37° and pH 6.8 or 7.5

Exp	pH	Duration of incubation (hr.)	P/N ratio of TCA precipitated proteins		
			Phosphorylated human serum proteins	Rephosphorized casein	Casein*
1	7.5	0	0.051	—	0.052
		24	0.041	—	0.048
		72	0.027	—	0.047
		147	0.024	—	—
	6.8	0	0.067	0.042	0.048
		24	0.049	0.039	0.047
		72	0.027	0.025	0.046
		147	0.022	0.021	—
	7.5	0	0.041	—	0.042
		142	0.018	—	0.039
		306	0.020	—	0.036

* The casein separated out from both buffer mixtures during the incubation, but samples were taken from the suspension after shaking.

Loss at 37° of P from phosphorylated serum proteins prepared with different POCl₃/protein ratios Different preparations of phosphorylated serum proteins showed differences in the rate of loss of combined P when the solutions were kept at 37° and it was thought that there might be qualitative differences in this respect between the phosphate groups most readily attached to the protein (i.e. by the use of lesser quantities of POCl₃) and those attached by more drastic phosphorylation

Human serum (20 ml) was phosphorylated with 0.14 g POCl₃ (i.e. a POCl₃/protein ratio of 0.1/1). The product was dialyzed against distilled water for 5 hr, and then against 0.9% NaCl until free from inorganic phosphate. The solution was brought to pH 7.5 with 0.2N NaOH and added to an equal volume of 0.75M NaHCO₃, H₂CO₃ buffer. Toluene was added and the mixture was kept in a sealed tube at 37°, samples being withdrawn after 141 and 260 hr for precipitation of the proteins with TCA.

The results (Table 4) showed that even with the 'lightly phosphorylated' proteins there was fairly rapid loss of P when the solution was kept at 37°.

Table 4 *Loss at 37° and pH 7.5 of P from phosphorylated human serum proteins prepared with a POCl₃/protein ratio of 0.1/1*

Duration of incubation (hr)	Type of precipitation*	P/N ratio
0	(Dialyzed liquid)	0.042
	HCl + acetone	0.040
	TCA	0.021
141	TCA	0.005
260	TCA	0.004

* The precipitates obtained with TCA were washed with ethanol and acetone.

A further experiment was made to cover a greater range of POCl₃/protein ratios, viz. 0.025–0.05/1. All these resulting phosphorylated human serum proteins, with P/N ratios ranging from 0.017 to 0.095, lost 80% of their combined P when the solutions were kept at 37° for 142 hr (Table 5). The possibility

Table 5 *Loss of P at 37° and pH 7.5 from phosphorylated human serum proteins prepared with different POCl₃/protein ratios*

POCl ₃ /protein ratio used in the preparation	P/N ratio	
	Dialyzed* solution	After 142 hr at 37° and subsequent dialysis*
0.025	0.017	0.005
0.05	0.024	0.005
0.10	0.038	0.008
0.20	0.055	0.010
0.30	0.076	0.015
0.50	0.095	0.023

* Dialysis at 4–6°

that some of the phosphorylated protein was split up into smaller molecules of P containing organic substances during the incubation, and that these smaller molecules subsequently passed through the dialyzing sac, was not explored.

Immunization with phosphorylated serum proteins

Two different antigens were used for immunization in the hope that antisera of differing serological specificity might be obtained. The 'high P' phosphorylated serum protein was prepared by the action of the usual great excess of POCl₃ on human serum proteins, and the 'low P' protein was obtained by incubating a solution of the 'high P' proteins at 39° and pH 7.5, with subsequent dialysis to remove free phosphate, incubation for 6 days removed about 6/7 of the combined P of the 'high P' phosphorylated proteins.

Precipitin tests carried out with the sera of the injected rabbits over a long period of immunization showed that all the rabbits had produced antibodies capable of reacting strongly with the antigens used for the injections (phosphorylated human serum proteins), with no marked difference between the results with the 'high P' and those with the 'low P' proteins. The antisera also gave strong or very strong precipitin reactions with untreated human serum, showing that phosphorylation had not destroyed the species specificity of the serum proteins. In a few cases only, the antisera reacted slightly with phosphorylated rabbit serum proteins, but no precipitate was obtained with phosphorylated chicken serum proteins or with phosphorylated gelatin. The results of some typical precipitin tests are given in Table 6.

DISCUSSION

The phosphorylated proteins obtained by the action of POCl₃ on serum proteins are readily precipitated by dilute acid (at pH 4–5 for the strongly phosphorylated proteins). In many other respects also, phosphorylation effects a distinct change in properties. With a moderate excess of phosphoryl chloride, the amount of P introduced into the proteins under the conditions of our experiments may correspond to as much as 50 phosphoryl groups/mol of protein, assuming a mean mol. wt. of 10⁵ for these proteins. Heidelberger *et al.* (1941), in similar investigations, found that crystalline egg albumin could take up 20–30 phosphoryl groups/mol. on the basis of a mol. wt. of 36,000 for egg albumin, and they noted that 'the introduction of these polar groups is accompanied by a remarkable series of changes in the chemical, physical and immunological properties of the protein'.

Table 6 *Precipitin reactions with antisera to phosphorylated human serum proteins*

Antigen		Dilution of antigen	Antisera to phosphorylated human serum proteins			
			'Low P'		'High P'	
			Rabbit no 461	Rabbit no 463	Rabbit no 480	Rabbit no 482
Human serum		1 20	+	+ ±	±	+ +
		1 100	+ + ±	+ + ±	+	+ + ±
		1 500	+ +	+ ±	±	+
		1 2500	±	tr	tr	+
Phosphorylated human serum proteins	'Low P'	1 20	+ ±	+ ±	tr	
		1 100	+ + ±	+ + ±	+	
		1 500	+ ±	+ ±	tr	
		1 2500	±	tr	tr	
	'High P'	1 20	+		tr	+ ±
		1 100	+ + ±		+	+ + ±
		1 500	+ ±		±	+ + ±
		1 2500	±		tr	+
Phosphorylated rabbit serum proteins	(a)*	1 20	-	-	-	-
		1 100	-	-	-	-
		1 500	-	-	-	±
		1 2500	-	-	-	-
	(b)*	1 20	-	-	-	-
		1 100	-	-	-	f tr
		1 500	-	-	-	tr
		1 2500	-	-	-	±

* The phosphorylated rabbit serum preparations (a) and (b) were obtained by the use of a POCl_3 /protein ratio of 0.5/1 and 2.5/1 respectively, both products were dialyzed free from inorganic phosphate

Precipitin readings - (no precipitate), f tr (faint trace), tr (trace), ±, +, + ±, + +, etc in increasing degrees of precipitation

In most of the experiments described in this paper we have used phosphorylated mixed serum proteins, the chief object being to prepare antigenic proteins containing ^{32}P , and a mixture of the phosphorylated serum globulins and albumins seemed most suitable for many of the serological tests. For some of the experiments reported here, however, and for others to be described later, purified and less complex mixtures of phosphorylated proteins have been used. Another difference between our experiments and those of Heidelberger *et al* (1941) is that in nearly all cases we have used considerably less phosphoryl chloride than was used by them, or by Rimington (1927). Our object was to ensure minimum loss of valuable ^{32}P in parallel experiments with 'radio' POCl_3 . Even with this smaller amount of phosphoryl chloride (0.5 g/g of protein as compared with the 2-3 g/g of protein used by Heidelberger *et al* and by Rimington in most of their experiments) the proteins were phosphorylated to a comparable degree for example, 1-1.5% of P, as compared with 1.52% for heavily phosphorylated egg albumin (Heidelberger *et al* 1941), and 0.71% for phosphorylated denatured serum globulin (Rimington, 1927). Furthermore, in one of our experiments it was found that a fivefold increase in the amount of phosphoryl chloride used did not lead to any significant increase in the amount of P attached to rabbit serum proteins.

The amount of P introduced into the serum proteins varied somewhat from one preparation to another, and the reason for this variation is not known. Freshly distilled POCl_3 was used in practically all our experiments, and the technique of phosphorylation was kept as constant as possible. Different samples of human serum may differ in their capacity to react with POCl_3 , but it is difficult to understand why, for example, a P/N ratio of 0.06-0.07 was obtained for the phosphorylated human serum albumin and globulin in one experiment (Table 1), whereas most subsequent experiments with serum proteins, and involving the use of the same relative amount of phosphoryl chloride, gave products with a P/N ratio of approximately 0.10. Similar variations were also noted by Heidelberger *et al* (1941) one of their phosphorylated egg albumin preparations contained 2.71% of P and had a P/N ratio of 0.18, as against P/N ratios of 0.104-0.115 for other preparations obtained by the same method of phosphorylation. It seems probable, as they suggest, that part of the P of phosphorylated proteins with a high P/N ratio is in a very labile form, and that variable amounts are split off when the experimental technique for isolating the phosphorylated protein is varied even slightly. We, like they, have found that this lability is very marked at 37°.

Of the various protein precipitants which we have used for the separation of the phosphorylated proteins, dilute acid (HCl, followed by final adjustment of the pH with acetic acid) has proved most satisfactory. Trichloroacetic acid was less satisfactory, and it is possible that the high acidity caused by this reagent (about pH 0.6 in the experiments referred to in Table 3) may have resulted in appreciable removal of P from the phosphorylated proteins. Where a solution of phosphorylated protein was required for immunological purposes, purification was effected by dialysis, followed by repeated acid precipitation where the preparation was required for injection.

The results of the immunological experiments showed that the antibodies produced by the injection of phosphorylated human serum proteins were not specific for the phosphoryl groups of the protein, for the antisera obtained gave little or no reaction with phosphorylated rabbit or chicken serum proteins. This result was rather surprising, for the introduction of an appreciable number of polar groups into the molecule of an antigenic protein usually leads to the acquisition of a new serological specificity characteristic for the introduced groupings. It is probable, however, that the phosphorylated human serum proteins rapidly lost most of their combined P after injection into rabbits, there was certainly no lack of antigenic power in the injected material, since powerful antibodies against the injected antigen (phosphorylated human serum proteins) were formed. This view is supported by the *in vitro* lability of the combined phosphate groups at 37° and pH 7.5 (Tables 3-5), incubation for several days resulted in a very marked decrease, sometimes as much as 80%, in the combined P of the proteins precipitable by trichloroacetic acid or those freed from dialyzable P containing compounds.

If the injected phosphorylated proteins rapidly lost their combined P, as the experiments described in this paper and in the related investigations with ³²P containing phosphorylated proteins (Banks, Bournsell, Dewey, Francis, Tupper & Wormald, 1948) suggest, the failure of the injected rabbits to produce antibodies to phosphorylated heterologous protein, and the production of antibodies capable of reacting with untreated human serum proteins, can readily be explained. It seems probable that our phosphorylated human serum protein preparations differed considerably, in their *in vivo* stability, from the phosphorylated egg albumin of Heidelberger *et al.* (1941), for the antisera produced by the injection of the latter antigen reacted only slightly with unchanged egg albumin. Heidelberger *et al.* obtained specific antibodies against phosphorylated egg albumin, but they observed that the new specificity conferred on egg albumin by the introduction of phosphoryl groups is not entirely a haptenic specificity in the sense defined by Landsteiner (1936).

The possibility of an enzymic removal of P from phosphorylated proteins, either by an enzyme present in our preparations of phosphorylated human serum proteins or in rabbit blood, cannot be excluded, but this possibility has

not as yet been investigated. We intend, however, to study the *in vivo* stability of phosphorylated human serum proteins, phosphorylated egg albumin and other phosphorylated proteins. It seems probable from the evidence already available that phosphorylated human serum proteins may not be the most suitable proteins for our investigations with ³²P containing protein antigens. For some of these purposes vitellin is more satisfactory (Francis & Wormald, 1948), but unfortunately it is almost insoluble in water when freed from firmly combined phosphatide.

The action of phosphoryl chloride on proteins may concern several groups of the protein, e.g. the hydroxyl groups of serine and threonine, and possibly the phenol group of tyrosine, the free amino group of lysine, and the guanidine group of arginine. Some of the attached phosphoryl groupings will, of course, be less firmly attached than others, and the existence of different P protein linkages might readily explain the considerable variations in the rate with which P is removed from phosphorylated proteins. It is difficult to understand, however, why this process is so rapid, particularly at 37°, in solutions kept at pH 7.5, for some phosphorylated amino acids are quite stable. Plimmer (1941), for example, found that the phosphoric esters of tyrosine, hydroxyproline, threonine, serine and isoserine were all stable to 0.5N or even N NaOH at 37°. It is our intention to study this problem further, with the aid of serological methods.

SUMMARY

- 1 Human serum proteins have been phosphorylated by phosphoryl chloride, and some of the chemical and immunological properties of the products have been studied.
- 2 These phosphorylated proteins lost appreciable amounts of their combined P when they were kept in solution at 37° and pH 7.5 or 6.8.
- 3 Immunization of rabbits with phosphorylated human serum proteins produced antibodies which reacted strongly with the injected proteins and also with untreated human serum proteins.
- 4 These antisera gave little or no precipitation with phosphorylated rabbit serum proteins or phosphorylated chicken serum proteins. No evidence, therefore, was obtained of the formation of antibodies specific for the phosphoryl group of phosphorylated proteins. It is probable, however, that the phosphorylated serum proteins rapidly lost a considerable fraction of their combined P after injection into the rabbits.

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POSTSCRIPT Since this paper was in the press we have seen the very valuable account of the chemical and immunological properties of phos

phorylated crystalline horse serum albumin given by Mayer & Heidelberger (1946) We hope to make full reference to this work later

REFERENCE Mayer, M & Heidelberger, M (1946) *J Amer chem Soc.* 68, 18

Studies in the Biochemistry of Micro-organisms

77 A SURVEY OF FUNGAL METABOLISM OF INORGANIC SULPHATES

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Clutterbuck, Mukhopadhyay, Oxford & Raistrick (1940), in a communication from this laboratory, described the results of a quantitative survey of the chlorine metabolism of 139 species or strains of moulds grown on Czapek-Dox 5% glucose solution containing 0.5 g KCl/l as the sole source of chlorine

The results are now given of a parallel quantitative survey of the sulphur metabolism of 115 species or strains of moulds grown on the same medium containing 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.01 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ /l as the sole source of sulphur

EXPERIMENTAL

Cultural conditions

The medium used throughout this investigation was the well known Czapek Dox solution of the following composition glucose, 50 g, NaNO_3 , 2.0 g, KH_2PO_4 , 1.0 g, KCl, 0.5 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g, distilled water, 1 l. All the constituents of the medium were of analytical reagent purity. Hence the sole source of sulphur was present as the sulphates of magnesium and iron, and amounted to 22.9 mg/flask (6.6 mg/100 ml) at the time of sowing. This medium (350 ml) was accurately measured into each of a number of 1 l conical flasks plugged with cotton wool, and sterilized by steaming for 0.5 hr on each of 3 consecutive days. The flasks were then inoculated with a pure culture of the chosen species of mould, and incubated at 24° for the requisite period of days.

The sulphate content of duplicate uninoculated flasks from each batch was determined by the method described later. The inoculated flasks were treated as follows. The mycelium from each species was separated quantitatively by filtration through a Buchner funnel, and was thoroughly washed with warm water. It was then dried to constant

weight *in vacuo*, weighed, ground and stored in a closely stoppered bottle prior to examination. The culture filtrate and washings were made up to a total volume of 500 ml, and analyzed by the methods described in the next section.

Analytical methods

I Sulphur as inorganic sulphate in culture filtrate

The concentration of inorganic sulphate present in the uninoculated medium is quite low (equiv. to 22.9 mg S/350 ml, or 4.58 mg/100 ml after dilution to 500 ml). The medium also contains relatively considerable amounts of nitrate, phosphate and chloride. Further, the culture filtrate resulting from the growth of different moulds may contain metabolic products of complex and unknown nature and may vary from almost colourless to deeply coloured. After a considerable amount of exploratory work, details of which will be published elsewhere, on a number of published methods the one finally chosen as being the most generally useful for our purpose was that based on Øllgaard's (1934) method. This method involves the precipitation of inorganic sulphate with benzidine in acetone and titration of the precipitate, after liberation of the sulphate with a little NaOH, with a standard BaCl_2 solution using sodium rhodizonate as indicator. Using this method we found that, in solutions containing 0.3 mg of sulphate S/10 ml portion, a precision of $\pm 1\%$ in duplicates can in general be assured, and of $\pm 5\%$ with certainty.

Reagents

- Trichloroacetic acid* 20% (w/v) solution in water
- Benzidine* 1% solution in pure acetone, freshly prepared each day
- Sodium hydroxide.* N NaOH, in water
- Ethanol-magnesia mixture* NH_4Cl (6 g) and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (6 g) in water (100 ml.) and made up to 1 l with absolute ethanol

Barium chloride 0.02N BaCl_2 , in water

Indicator A freshly prepared aqueous solution of sodium rhodizonate of a fairly deep orange colour

All reagents should be A.R.

Method The solution (20 ml) to be examined is acidified with trichloroacetic acid (10 ml). Any precipitate formed is separated by filtration. Duplicate 10 ml samples of the clear solution are pipetted into 50 ml centrifuge tubes. Benzidine solution in acetone (25 ml) is added to each tube in a fine stream, and the tubes are capped and allowed to stand for at least 0.5 hr. They are then centrifuged at medium speed for 15–20 min and the clear supernatant fluid is carefully decanted. The tubes are now inverted on filter paper to drain residual acetone. It was found by control experiments that washing of the precipitate with acetone is unnecessary, and that even when the culture filtrate is highly coloured the precipitate left after decantation is almost white, and the amount of residual colouring matter was never sufficient to interfere with subsequent titration. The precipitate is now prepared for titration by dissolving in 0.1 ml of N NaOH. Shaking and warming facilitate solution. Ethanolic magnesia mixture (10 ml) is added to the solution to precipitate phosphates, and the tubes are kept at a temperature of 65–70° in a thermostat during the titration. Sufficient sodium rhodizonate solution is added to give a definite yellow colour to the solution, and care must be taken to add the same amount of indicator to each tube of a series of estimations. 0.02N BaCl_2 is added drop by drop from a micro burette graduated in 0.01 ml. During the titration the contents of the tube are rotated gently. The yellow colour changes through orange and reddish orange to a rather bright red. The BaCl_2 should be added carefully until there is a definite and permanent blue tint in the red, continuing heating in the thermostat and further addition of BaCl_2 if necessary. Illumination needs to be good, a blue glass electric light placed close to the tube and a white background help considerably.

1 ml of 0.02N BaCl_2 = 0.3207 mg S

Of the 119 species and strains of moulds examined only four, which were discarded, gave trouble and then only after a very long period of incubation. In three cases, i.e. *Penicillium frequentans* Westling, P. 28, *P. luteum* Zukal, P. 42, *P. rugulosum* Thom, P. 50, the interference was due to the extreme viscosity of the culture filtrate, and in the fourth, *Aspergillus niger* van Tieghem, no. 33, a heavy precipitate prevented titration.

II Sulphur as total inorganic sulphate in culture filtrate after heating with 0.3N HCl

The method commonly used for the hydrolysis of the ethereal sulphates in urine has been modified and applied to the mould culture filtrates. A portion of the culture filtrate is accurately measured into a conical flask and acidified with $\frac{3}{4}$ vol. pure conc. HCl, so that the final concentration of HCl is approx. 0.3N. A small funnel and watch glass are inserted in the neck of the flask to lessen evaporation, and its contents are boiled gently for 30 min. After cooling, the solution is transferred to a measuring flask, a little bromocresol green is added and the solution neutralized with NaOH. The total inorganic sulphate now present is determined in portions by the method described in § I. Reagents are checked by means of a blank experiment. Phenyl potassium sulphate, initially free from inorganic sulphates, gave a 98.2% yield of inorganic sulphate when estimated by this method.

III Sulphur as inorganic sulphate generated in culture filtrate after heating with 0.3N HCl

This is obtained by subtracting the value given by the estimation in § I from that obtained as described in § II.

IV Total sulphur in culture filtrate

To a sample of the culture filtrate (usually 100 ml) is added fuming HNO_3 (10 ml) and KNO_3 (0.5 g). The mixture is evaporated almost to dryness in a Kjeldahl flask, and is then treated exactly as in the estimation of total sulphur in the mycelium described in § VI.

V Sulphur in culture filtrate not present as inorganic sulphate after heating with 0.3N HCl

This is obtained by subtracting the value given by the estimation in § II from that obtained as described in § IV.

VI Total sulphur in the mycelium

About 1 g of dried and powdered mycelium is accurately weighed, and is gently digested for 4 hr in a Kjeldahl flask with fuming HNO_3 (10 ml) and KNO_3 (0.5 g). The acid is then removed in a stream of air on a water bath. A further 10 ml fuming HNO_3 are added, the contents heated for 2 hr and the acid removed as before. The residue is now digested on a sand bath for 1–2 hr with H_2O_2 (10 ml of 100 vol.) added at intervals 2 ml at a time. The clear solution is evaporated on the water bath, taken up in 0.25N-HCl (20 ml) and evaporated to dryness. Evaporation with HCl is repeated, and the final white residue is dissolved in 100 ml of a mixture of water and trichloroacetic acid (final acid concentration, 6%). The amount of inorganic sulphate is estimated in 10 ml. samples by precipitation with benzidine as described in § I.

VII Residual glucose in the culture filtrate

This is estimated polarimetrically.

RESULTS

The utilization of sulphur initially present as inorganic sulphate, by 115 mould species or strains

In this part of the investigation only the culture filtrate was examined.

Two flasks, each containing 350 ml of Czapek Dox culture medium with a sulphate content equivalent to 22.9 mg of sulphur/flask, were inoculated at the same time and from the same culture of the species or strain of mould being examined. One flask was in general, harvested after an incubation period of 17–25 days, after which time the residual glucose was usually between 0.1 and 1.0%. The second flask was harvested after a much longer incubation period, usually 5–10 weeks, by which time the glucose had either been completely metabolized, or its utilization had become extremely slow. The residual inorganic sulphate was estimated in each flask by the method described above.

The results obtained are given in Table 1. The figures given in column 5 show the percentage utilization of sulphate, so that a figure of 97.6% means that only 2.4% of the sulphur originally present in the culture medium as inorganic sulphate remains in that form in the culture filtrate. The moulds examined are listed in descending order of

Table 1 Utilization of inorganic sulphate by 115 species and strains of fungi

Organism	Catalogue no *	Incubation period (days)	Residual glucose by polarimeter (g /100 ml)	Utilization of SO ₄ (%)
Species of <i>Penicillium</i>				
<i>P. puberulum</i> Bainier	P 47	24	0.2	97.6
		38	0.0	96.0
<i>P. notatum</i> Westling	P 45	23	0.7	95.6
		38	0.0	96.4
<i>P. citrinum</i> Thom	P 27	21	0.6	85.3
		39	0.1	70.3
<i>P. olivino viride</i> Biourge	P 46	24	0.2	80.6
		37	0.2	97.4
<i>P. brunneo rubrum</i> Dierckx	P 8	21	0.4	80.3
		58	0.0	58.0
<i>P. lanosum</i> Westling	P 86	21	1.0	80.2
		68	0.0	59.6
<i>P. candido fulvum</i> Dierckx	P 12	18	3.6	79.6
		65	0.1	72.0
<i>P. chrysogenum</i> Thom	P 57	25	0.0	79.1
		39	0.0	40.6
<i>P. phaeo janthinellum</i> Biourge	P 91	22	0.1	76.6
		56	0.0	60.4
<i>P. griseo brunneum</i> Biourge	P 36	22	0.2	76.1
		37	0.0	45.8
<i>P. Hagemi</i> Zaleski	P 84	21	0.3	74.0
		68	0.0	55.8
<i>P. citreo nigrum</i> Dierckx	P 20	21	1.2	73.4
		57	0.0	96.6
<i>P. citrinum</i> Thom	P 26	24	0.1	73.4
		56	0.0	55.7
<i>P. digitatum</i> Saccardo	P 33	43	0.0	73.0
		66	0.5	79.7
<i>P. aurantio brunneum</i> Dierckx	P, 2	20	0.8	68.8
		60	0.0	95.2
<i>P. citreo viride</i> Biourge	P 24	24	0.3	68.8
		55	0.0	68.4
<i>P. commune</i> Thom	P 30	21	0.4	67.8
		38	0.0	50.7
<i>P. patris mei</i> Zaleski	P 90	22	0.3	66.6
		68	0.0	50.6
<i>P. solitum</i> Westling	P 52	24	0.6	66.1
		39	0.1	60.9
<i>P. caseicolum</i> Bainier	P 11	18	2.0	66.0
		60	0.0	33.9
<i>P. citreo roseum</i> Dierckx	P 22	21	0.7	65.5
		57	0.0	98.1
<i>P. chloro leucon</i> Biourge	P 14	18	0.4	64.8
		55	0.1	52.8
<i>P. chloro-phaeum</i> Biourge	P 15	18	1.4	64.8
		57	0.0	70.5
<i>P. ochraceum</i> Bainier	P 88	22	0.4	63.5
		68	0.0	47.2
<i>P. baculatum</i> Westling	P 7	21	0.3	63.4
		59	0.0	58.0
<i>P. chrysitis</i> Biourge	P 16	18	3.1	60.5
		61	0.0	58.4
<i>P. griseo-roseum</i> Dierckx	P 39	22	0.8	60.4
		39	0.2	38.9
<i>P. flavido marginatum</i> Biourge	P 78	20	0.1	59.8
		57	0.1	26.2

* London School of Hygiene and Tropical Medicine (L S H T M)

Table 1 (cont)

Organism	Catalogue no *	Incubation period (days)	Residual glucose by polarimeter (g/100 ml)	Utilization of SO ₄ (%)
<i>P. Bialowiezensis</i> Zaleski	P 71	19 56	0.2 0.0	59.7 35.9
<i>P. citreo roseum</i> Dierckx	P 23	24 55	0.4 0.1	59.2 61.1
<i>P. citrinum</i> Thom	P 6	20 60	1.1 0.0	58.5 58.4
<i>P. fusco-glaucum</i> Biourge	P 81	21 56	0.5 0.0	58.1 82.3
<i>P. chrysogenum</i> Thom	P 10	21 55	0.4 0.0	57.7 60.5
<i>P. frequentans</i> Westling	P 29	21 39	0.8 0.0	56.3 80.1
<i>P. griseo-fulvum</i> Dierckx	P 38	22 43	2.0 0.7	54.1 48.9
<i>P. citreo nigrum</i> Dierckx	P 21	21 61	2.2 0.2	53.7 56.8
<i>P. Raciborski</i> Zaleski	P 92	22 67	0.1 0.0	52.8 42.8
<i>P. stilton</i> Biourge	P 54	25 64	2.0 0.1	52.8 62.8
<i>P. citreo sulfuratum</i> Biourge	P 66	18 71	2.3 0.0	50.7 66.2
<i>P. expansum</i> Lank	P 41	23 43	1.1 0.1	50.2 95.8
<i>P. biforme</i> Thom	P 72	19 67	0.1 0.1	49.8 24.5
<i>P. citreo sulfuratum</i> Biourge	P 59	26 65	1.7 0.1	49.7 52.0
<i>P. cinerascens</i> Biourge	P 65	18 71	2.8 1.0	48.5 52.0
<i>P. spinulosum</i> Thom	P 96	25 67	0.1 0.0	48.0 39.8
<i>P. citrinum</i> Thom	P 25	24 55	0.3 0.0	47.4 65.6
<i>P. cyaneo fulvum</i> Biourge	P 34	22 66	1.2 0.5	45.4 55.0
<i>P. Oledzki</i> Zaleski	P 89	22 69	2.4 0.0	44.5 60.9
<i>P. Siemaszki</i> Zaleski	P 95	25 69	1.0 0.1	44.1 31.0
<i>P. sublateritium</i> Biourge	P 55	25 42	0.3 0.0	42.8 60.9
<i>P. purpurogenum</i> Stoll	P 48	24 39	0.3 0.1	40.6 45.4
<i>P. viridi-dorsum</i> Biourge	P 56	25 42	1.3 0.1	40.6 43.7
<i>P. fellutanum</i> Biourge	P 67	19 56	0.5 0.0	40.2 48.5
<i>P. Dierckxi</i> Biourge	P 32	22 43	2.0 0.1	39.7 61.2
<i>P. roqueforti</i> Thom	P 93	22 69	3.1 0.0	37.6 40.6
<i>P. cyaneo fulvum</i> Biourge	P 31	21 65	1.5 0.5	37.5 50.7
<i>P. aurantio-griseum</i> Dierckx var <i>poznanensis</i> Zaleski	P 69	19 56	0.2 0.0	36.3 55.0
<i>P. Niklewski</i> Zaleski	P 61	26 65	0.9 0.1	36.2 37.6

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Table 1 (cont)

Organism	Catalogue no *	Incubation period (days)	Residual glucose by polarimeter (g /100 ml)	Utilization of SO ₄ (%)
<i>P. obscurum</i> Biourge	P 87	21 68	1.6 0.0	35.8 50.7
<i>P. fellutanum</i> Biourge	P 60	26 65	2.1 0.1	35.4 50.2
<i>P. Biourgei</i> Dierckx	P 73	20 67	1.8 0.0	35.0 39.8
<i>P. roseo citreum</i> Biourge	P 94	25 69	1.4 0.9	34.1 37.2
<i>P. Adametzi</i> Zaleski	P 58	25 64	2.5 0.1	34.0 58.2
<i>P. granulatum</i> Bainier	P 35	22 38	0.4 0.2	32.8 45.4
<i>P. flavi-dorsum</i> Biourge	P 79	20 70	2.2 0.0	32.4 43.2
<i>P. gorgonzola</i> Weidemann	P 83	21 69	2.6 0.1	32.3 53.3
<i>P. subcinereum</i> Westling	P 98	25 69	2.8 0.1	32.0 38.4
<i>P. terlikowski</i> Zaleski	P 63	18 67	2.5 0.2	30.1 54.2
<i>P. decumbens</i> Thom	P 76	20 71	2.9 0.0	29.4 40.6
<i>P. chrysogenum</i> Thom	P 18	21 60	2.4 0.1	29.2 53.3
<i>P. brunneo rubrum</i> Dierckx	P 9	18 63	2.9 0.8	28.8 50.1
<i>P. frequentans</i> Westling	P 80	20 70	3.2 0.0	28.8 44.2
<i>P. chrysogenum</i> Thom	P 17	21 61	3.3 1.5	27.6 34.4
<i>P. frequentans</i> Westling	P 82	21 70	3.1 0.1	27.1 65.4
<i>P. carmino violaceum</i> Dierckx	P 64	18 71	3.3 0.0	26.7 50.7
<i>P. banolium</i> Biourge	P 70	19 67	1.4 0.0	25.8 43.2
<i>P. Paczoslui</i> Zaleski	P 62	18 71	2.5 0.0	25.0 45.0
<i>P. aurantio virens</i> Biourge	P 4	20 107	3.5 0.0	22.9 32.0
<i>P. meleagrinum</i> Biourge	P 43	23 78	3.4 2.2	22.7 36.7
<i>P. atramentosum</i> Thom	P 1	20 64	3.4 0.1	21.7 47.7
<i>P. griseo fulvum</i> Dierckx	P 68	19 71	3.5 0.0	20.1 74.8
<i>P. flavo-cinereum</i> Biourge	P 77	20 70	3.5 0.1	19.6 44.5
<i>P. spiculisporum</i> Lehman	P 53	25 43	1.6 0.1	17.0 20.0
<i>P. schneegii</i> Boas	P 51	24 64	4.2 2.4	12.4 32.8
<i>P. minus luteum</i> Dierckx	P 44	23 78	3.8 1.8	11.7 28.0
<i>P. jantho citrinum</i> Biourge	P 40	23 78	3.8 3.2	10.5 25.8
<i>P. aurantio-candidum</i> Dierckx	P 3	20 65	4.5 3.6	7.0 42.6
<i>P. brunneo violaceum</i> Biourge	P 10	18 61	2.8 0.2	5.8 73.4

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Table 1 (cont)

Organism	Catalogue no *	Incubation period (days)	Residual glucose by polarimeter (g /100 ml)	Utilization of SO ₄ (%)
Species of <i>Aspergillus</i>				
<i>A. fumigatus</i> Fresenius	No 47	19	2.4	99.3
		47	0.3	85.1
<i>A. terreus</i> Thom	Am 1	17	0.5	93.0
		59	0.0	82.5
<i>A. terreus</i> Thom	No 3	17	0.6	90.5
		59	0.0	98.2
<i>A. terreus</i> Thom	Ab 2	19	0.4	85.5
		57	0.0	97.2
<i>A. terreus</i> Thom	No 37	17	0.8	75.6
		59	0.0	99.5
<i>A. flavus</i> Link	No 51	19	0.5	74.8
		47	0.2	98.7
<i>A. terreus</i> Thom	Ac 24	17	0.5	71.1
		57	0.6	100.0
<i>A. Sydowi</i> Bainier & Sartory	No 43	18	0.6	68.5
		46	0.0	88.5
<i>A. terreus</i> Thom	Am 2	19	0.2	67.1
		57	0.0	97.7
<i>A. ochraceus</i> Wilhelm	No 69	19	1.5	66.2
		47	0.2	97.9
<i>A. terreus</i> Thom	Ab 1	19	0.8	61.0
		59	0.0	69.9
<i>A. terreus</i> Thom	Ac 107	17	0.3	58.5
		57	0.0	63.9
<i>A. oryzae</i> (Ahlburg) Cohn	A 11	25	0.5	57.0
		48	0.3	50.7
<i>A. Wentii</i> Wehmer	No 2	19	2.0	42.8
		46	0.6	37.1
<i>A. nidulans</i> Eidam	No 46	18	1.6	41.4
		46	0.1	69.0
<i>A. versicolor</i> Vuill. (Tiraboschi)	No 18	19	2.2	41.0
		46	0.1	62.2
<i>A. terreus</i> Thom	Ac 100	17	2.6	31.6
		60	0.0	57.9
<i>A. tamaris</i> Kita	No 55	20	3.4	19.7
		47	0.5	42.8
<i>A. candidus</i> Link	No 32	25	3.8	17.9
		49	0.1	44.5
Miscellaneous species				
<i>Clasterosporium</i> sp	Ag 64	18	0.7	39.7
		46	0.1	42.0
<i>Stysanus stemonites</i> (Pers.) Corda	No 137	20	2.4	33.2
		48	0.1	41.0
<i>Stemphylium lanuginosum</i> Harz	No 56	20	4.2	31.5
		47	2.5	21.8
<i>Syncephalastrum cinereum</i> Bainier	No 112	20	4.4	22.7
		48	0.4	36.2
<i>Absidia spinosa</i> Lendner	No 118	20	4.0	19.7
		48	0.6	29.7
<i>Alternaria</i> sp	Ag 17	20	4.3	11.8
		43	3.3	24.0
<i>Trichoderma viride</i> Pers. ex Fries	No 85	19	4.1	11.3
		48	1.8	24.0
<i>Caldariomyces fumago</i> Woronichin	Ag 92	18	2.9	7.0
		46	1.5	68.4
<i>Cephalosporium</i> sp	Ag 30	25	4.3	4.8
		49	3.1	14.4

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the amount of sulphate metabolized after the shorter incubation period. Eighty seven species or strains of *Penicillium*, 19 of *Aspergillus* and 9 miscellaneous species were examined

Sulphur balance sheets of fourteen species or strains of moulds

The aims of this part of the investigation which, because of the large number of estimations involved, was of necessity somewhat restricted, were twofold

(a) To explore the variations in sulphur metabolism of different strains within a single species of mould. For this purpose six authentic strains of *Aspergillus terreus* Thom were chosen, since it was already known, from investigations carried out in this laboratory, that different strains of this species give rise to a large number of different metabolic products. Two of these strains, no. 45 and Am 2, metabolize considerable amounts of inorganic chloride (Clutterbuck *et al.* 1940)

(b) To explore the variations in sulphur metabolism of different species of moulds within a single genus. Seven different species of *Penicillium* were chosen for this purpose

Eight flasks, each containing 350 ml of Czapek Dox medium, were sterilized, inoculated with the species or strain under investigation, and incubated at 24°. Four flasks of each strain or species were harvested, and the filtered contents made up to 2 l after (a) substantially all the glucose had been utilized, and four flasks after (b) extended incubation for 9–11 weeks. In the culture filtrate, residual inorganic sulphate, sulphate generated after acid hydrolysis and total sulphur in solution were determined, and total sulphur in the mycelium, by the methods previously described. The results obtained are given in Table 2

DISCUSSION

An outstanding fact, that emerges from the results presented in Table 1, is the evident importance of sulphur in the metabolism of moulds, as indicated by the large amounts of inorganic sulphate absorbed from the nutrient medium. Thus, of the 115 species or strains examined, seven species of *Penicillium* and nine species or strains of *Aspergillus*, including six strains of *A. terreus* Thom, utilized, after either the shorter or longer incubation period, 90.0% or more of the inorganic sulphate originally present in the nutrient medium. Similarly, 40 species or strains utilized from 60.0 to 89.9% of the inorganic sulphate. With 29 species or strains the percentage utilization of inorganic sulphate was more than 5% greater after the shorter period of incubation than after the longer period. In some of these cases, where the difference is relatively small, it is possible that the difference represents variations between single flasks. In others, it is probably due to the hydrolysis of 'etheral sulphates' formed as metabolic products (see below)

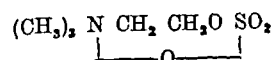
The seven different species of *Penicillium*, six different strains of *Aspergillus terreus* Thom and one of *A. fumigatus* Fresenius, showing a large percentage utilization of inorganic sulphate, were chosen for a more detailed examination of their sulphur metabolism with the results given in Table 2. Consideration of these results leads to the following conclusions

(1) *Utilization of inorganic sulphate* (column 6, Table 2). All seven species or strains of *Aspergillus* and three species of *Penicillium* utilized the whole of the inorganic sulphate originally present in the medium within the limits of experimental error $\pm 5\%$, and after either the shorter or longer period of incubation. Two of the four remaining species utilized over 90%. The results are thus in substantial agreement with those given in Table 1

(2) *Inorganic sulphate generated after acid hydrolysis* (column 8, Table 2). With most species or strains examined the amount of inorganic sulphate re-generated after acid hydrolysis under relatively mild conditions (boiling with 0.3N-HCl for 30 min) is considerable, reaching a maximum of 60.1% with *Penicillium citreo-roseum*, and exceeding 25% with ten other species or strains. The nature of this sulphur has not yet been established, but it is clear that it is present in some relatively labile form possibly as 'etheral sulphates'

The presumed formation of 'etheral sulphates' does not, however, appear to be a property possessed by all the species examined. Thus the percentage amount of 'etheral sulphates' formed by *P. notatum* (5.3 and 3.9% after 35 and 75 days' incubation respectively), and by *P. aurantio-brunneum* (5.2% after 36 days' incubation) falls within, or very close to, the experimental error of the method of estimation ($\pm 5\%$). A similar observation was made by Rippel & Behr (1936) who cultivated *Aspergillus niger* on a chemically defined medium, somewhat different in nature to Czapek Dox medium, and containing the sulphates of potassium, magnesium and zinc. They observed the utilization of inorganic sulphate, and showed that boiling the metabolism solution with 0.5N- or N-HCl for 2 hr gave no appreciable increase in inorganic sulphate. We have confirmed the absence of 'etheral sulphate' formation by our strain of *A. niger* L.S.H.T.M. strain Ac 1 when grown on Rippel & Behr's medium

So far as we are aware only one 'etheral sulphate' has been isolated in a state of purity from mould cultures. Woolley & Peterson (1937) extracted cyclic choline sulphate



(β sulphatoethyltrimethylammonium betaine)

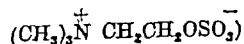


Table 2 Balance sheet showing distribution of sulphur originally supplied as inorganic sulphate and metabolized by fourteen species or strains of moulds

Organism	Catalogue no *	Incubation period (days)	Residual glucose (g/100 ml)	Weight of mycelium (g/flask)	Residual inorganic SO ₄ I (%)	Total SO ₄ after hydrolysis II (%)	SO ₄ generated after hydrolysis III (%)	Total S in filtrate IV (%)	Non SO ₄ S after hydrolysis V (%)	S in mycelium VI (%)	Total S accounted for IV + VI (%)
<i>A. terreus</i>	Ac 24	20 71	0.2 —	3.01 2.18	0.0 0.5	26.8 16.2	26.8 15.7	60.0 70.8	40.1 54.6	32.2 21.8	90.1 92.6
<i>A. terreus</i>	No 45	29 78	0.2 —	3.19 1.92	0.0 0.0	19.8 35.5	19.8 35.5	52.8 74.7	33.0 39.2	47.2 29.4	100.0 104.1
<i>A. terreus</i>	No 3	22 71	0.1 —	5.78 3.41	2.0 0.5	18.5 27.1	15.6 20.6	37.4 48.5	18.9 21.4	52.6 40.0	90.0 88.5
<i>A. terreus</i>	Ab 2	33 62	0.1 —	5.45 4.56	12.0 0.0	35.5 43.5	23.5 43.5	49.4 53.8	13.9 10.3	46.3 47.6	95.6 101.4
<i>A. terreus</i>	No 37	30 63	0.1 —	0.04 4.73	14.0 0.0	27.2 39.0	13.2 30.0	41.0 51.5	13.8 12.5	56.8 44.6	97.8 90.1
<i>A. terreus</i>	Am 2	30 74	0.1 —	3.40 2.57	19.0 1.2	35.4 27.8	16.4 20.6	53.7 49.8	18.3 22.0	40.3 37.6	94.0 87.4
<i>A. fumigatus</i>	No 47	41 76	1.5 —	3.00 3.35	0.0 0.7	16.5 16.5	16.5 15.8	48.1 43.2	31.6 26.7	37.9 50.3	80.0 93.5
<i>P. citreus nigrum</i>	P 20	41 74	0.0 —	2.49 2.15	0.0 3.5	40.6 45.0	40.6 41.5	59.9 70.0	19.3 25.0	32.7 24.0	92.6 94.0
<i>P. chrysogenum viride</i>	P 46	35 70	0.0 —	2.48 1.06	0.6 0.5	52.1 49.4	45.5 42.9	83.9 77.8	31.8 28.4	15.9 22.6	99.8 100.4
<i>P. puberulum</i>	P 47	27 70	0.0 —	2.79 1.79	4.0 0.0	36.1 20.6	31.2 20.6	64.6 68.1	28.5 47.5	28.5 27.2	93.1 95.3
<i>P. expansum</i>	P 41	41 68	0.4 —	2.13 1.07	34.0 28.2	63.1 62.5	29.1 34.3	75.6 77.4	12.5 14.0	23.1 26.4	98.7 103.8
<i>P. citreus roseum</i>	P 22	30 67	0.2 —	3.05 3.30	21.8 4.5	43.6 61.6	21.8 60.1	59.0 72.1	15.4 7.5	40.6 31.4	99.6 103.5
<i>P. notatum</i>	P 45	35 75	0.0 —	2.20 1.75	0.4 8.4	5.7 12.3	5.3 3.9	70.0 99.6	64.3 84.3	24.8 7.4	94.8 104.0
<i>P. aurantio-brunneum</i>	P 2	30 68	0.0 —	3.98 2.80	23.4 17.4	28.6 20.8	5.2 9.4	45.9 49.8	17.3 23.0	43.3 50.6	89.2 100.4

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as colourless crystals from the mycelium of *A. Sydowii* Bamier & Sartory grown on a glucose mineral salts medium. This substance yields choline and sulphuric acid on acid hydrolysis.

(3) *Sulphur in the culture filtrate not present as inorganic sulphate after acid hydrolysis* (column 10, Table 2). All the species and strains examined convert considerable amounts of inorganic sulphate into water soluble, sulphur-containing compounds, which do not give rise to inorganic sulphates on mild acid hydrolysis. In some cases the conversion is surprisingly large, e.g. *Penicillium notatum*, 75 days' incubation, 84.3%, *Aspergillus terreus*, strain Ac. 24, 71 days' incubation, 54.6%, *Penicillium puberulum*, 70 days' incubation, 47.5%.

Nothing is known at present as to the nature of these sulphur compounds, though it is probable that the sulphur is in organic combination. Hydrogen sulphide and mercaptans were not encountered, although Birkinshaw, Findlay & Webb (1942) established the formation of methyl mercaptan by the wood-rotting fungus *Schizophyllum commune* Fr., when grown on a synthetic medium containing glucose with ammonium and magnesium sulphates as sources of sulphur.

It is improbable that the sulphur is present to any considerable extent as protein or peptone. Thus, although Clutterbuck, Lovell & Raistrick (1932) isolated from culture filtrates of strains of *Penicillium chrysogenum* Thom and *P. notatum* Westling, including the strain P. 45, a protein containing 3.5% of cystine, the yield, expressed as a percentage of the inorganic sulphate in the medium, is small. Similarly, Rippel & Behr (1936) state that, of the inorganic sulphate utilized by *Aspergillus niger*, less than one seventh is present as protein and none as peptone.

Very few mould metabolic products containing sulphur have been isolated in a state of purity. It may be significant, however, that the different penicillins, metabolic products of *Penicillium notatum*, all give $\beta\beta$ -dimethylcysteine on hydrolysis, and that another antibiotic, gliotoxin, produced by a variety of fungi, including *Aspergillus fumigatus* Fresenius (Menzel, Wintersteiner & Hoogerheide, 1944) and *Penicillium cinerascens* Biourge (Bracken & Raistrick, 1947), has the empirical formula $C_{13}H_{14}O_4N_2S_2$.

Ovcharov (1937) has demonstrated qualitatively the formation of thiourea by cultures of *Verticillium albo-atrum* and *Botrytis cinerea* grown on Richards's (1897) medium, containing magnesium and ferrous sulphates as sole source of sulphur.

(4) *Sulphur in mycelium* (column 11, Table 2). Although the total sulphur in the mycelium amounts in some cases, particularly with strains of *Aspergillus terreus*, to about half that supplied originally as inorganic sulphate, the percentage of sulphur in the dried mycelium is, with three exceptions, fairly

uniform, and is consistently small. The percentage sulphur content in the dried mycelium of the different species and strains examined is as follows (d = incubation period in days): *A. terreus*, Ac. 24 29d, 0.24%, 71d, 0.23%; *A. terreus*, no. 45 29d, 0.34%, 78d, 0.35%; *A. terreus*, no. 3 22d, 0.21%, 71d, 0.27%; *A. terreus*, Ab. 2 33d, 0.20%, 62d, 0.24%; *A. terreus*, no. 37 30d, 0.22%, 63d, 0.22%; *A. terreus*, Am. 2 30d, 0.27%, 74d, 0.33%; *A. fumigatus*, no. 47 41d, 0.29%, 76d, 0.34%; *Penicillium citreo nigrum*, P. 20 41d, 0.30%, 74d, 0.25%; *P. olivino viride*, P. 46 35d, 0.15%, 70d, 0.26%; *P. puberulum*, P. 47 27d, 0.23%, 70d, 0.35%; *P. expansum*, P. 41 41d, 0.25%, 68d, 0.31%; *P. citreo roseum*, P. 22 36d, 0.25%, 67d, 0.22%; *P. notatum*, P. 45 35d, 0.25%, 75d, 0.10%; *P. aurantio brunneum*, P. 2 36d, 0.25%, 68d, 0.41%.

Thus, although the maximum and minimum weights of dried mycelium/flask for the shorter incubation period are 6.04 g (*Aspergillus terreus*, no. 37, 30 days) and 2.13 g (*Penicillium expansum*, P. 41, 41 days), their respective sulphur contents are 0.22 and 0.25%.

The dry weight of mycelium (Table 2, column 5) is less after the longer period of incubation than after the shorter period, in every case except for *Aspergillus fumigatus*, no. 47, clearly indicating that autolysis has taken place in the interval. Disregarding any results which fall within the limits of experimental error ($\pm 5\%$) there has been during this interval:

(a) A loss of sulphur from the mycelium to the metabolism solution in seven cases (limits -8.7% with *Penicillium citreo nigrum*, P. 20, to -17.8% with *Aspergillus terreus*, no. 45) and a gain in three cases (limits $+6.7\%$ with *Penicillium olivino viride*, P. 46, to $+12.4\%$ with *Aspergillus fumigatus*, no. 47).

(b) A gain in 'etheral sulphates' in the metabolism solution in seven cases (limits $+5.2\%$ with *Penicillium expansum*, P. 41, to $+38.3\%$ with *P. citreo roseum*, P. 22) and a loss in two cases (-10.6% with *P. puberulum*, P. 47, and -11.1% with *Aspergillus terreus*, Ac. 24).

(c) A gain in sulphur compounds, other than 'etheral sulphates', in the metabolism solution in six cases (limits $+5.7\%$ with *Penicillium citreo nigrum*, P. 20, and *P. aurantio brunneum*, P. 2, to $+20.0\%$ with *P. notatum*, P. 45) and a loss in one case of -7.9% with *P. citreo roseum*, P. 22.

SUMMARY

I. A survey has been made of the utilization of inorganic sulphate by 115 species or strains of moulds, grown on Czapek-Dox glucose solution con-

taining magnesium and ferrous sulphates as the sole source of sulphur

2 This has demonstrated the universal importance of inorganic sulphates in the metabolism of moulds, since all species or strains tested utilized 10 % or more, 56 utilized 60 % or more, and 16 over 90 % of the inorganic sulphate in the medium

3 A more detailed examination of the sulphur

metabolism of seven species of *Penicillium*, six strains of *Aspergillus terreus* Thom, and one strain of *A. fumigatus* Fresenius, grown on the same medium, revealed the fact that some of them form considerable amounts of 'etheral sulphates' while others, particularly *Penicillium notatum* Westling, give large yields of other, probably organic, sulphur compounds, of at present unknown nature

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Studies on Ruminant Saliva

1 THE COMPOSITION AND OUTPUT OF SHEEP'S SALIVA

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In all domestic animals the principal function of saliva is that of a lubricant to assist mastication and deglutition. In ruminants however, saliva has a still more important role, for the rumen has no secretion of its own and the saliva forms a fluid medium for the transport of ingesta both back to the mouth for remastication and onwards through the stomach to the small intestine. It forms also a buffered medium in which the micro organisms of the rumen can flourish. Particular interest therefore is attached to the saliva of the ruminant, to its composition and the amounts in which it is formed.

The literature on ruminant saliva is not very extensive except for a few more recent papers, most of it has been reviewed by Mangold (1929). The sublingual and submaxillary glands, when stimulated

by eating, secrete a saliva of low ash and alkalinity which contains mucin and whose function is limited to lubricating the bolus of food. The parotid gland, on the other hand, secretes during eating and rumination, at a greater rate on the side on which the bolus is being masticated, and its flow continues at a reduced rate during the intervals of rest. The parotid saliva is formed in large amounts, it contains no ptyalin but has a high alkalinity and buffering power. It is consequently the most important secretion for the maintenance of normal rumen function.

The output of saliva by various ruminants as reported in the literature is summarized in Table 1. The quantity produced per day has been estimated at 56 kg for the ox by Cohn (1886) and at 50 l for the

Table 1. *Published values for outputs of ruminant saliva*

Species	Secretion	Output of saliva		Reference
		ml/hr	Total/day	
Ox	Parotid	800-2400	56 kg	Cohn (1886)
Cow	Mixed	—	50 l.	Markoff (1913)
Sheep	Parotid	25	—	Scheunert & Trautmann (1921)
	Parotid	3.6-21.6	—	Popov (1921)*
Goat	Parotid	10	—	Do
	Parotid	60	—	Babichev (1925)*

* Quoted by Babkin (1944)

Table 2 Published composition of ruminant saliva*

Animal	Secretion	Content (g/100 ml)				Content (mg/100 ml)							CO ₂ content (ml/100 ml)	pH	Reference
		Dry matter	Ash	Alkalinity as soda		N	Na	K	Ca	Mg	P	Cl			
Sheep	Parotid	1.1-1.25	0.75-0.9	0.58-0.77		—	—	—	—	—	—	—	—	—	Scheunert & Trautmann (1921)
		—	—	0.58-0.77		—	—	—	—	—	—	—	—	—	Schwarz & Rasp (1926)
	Mixed	—	1.2	—		—	303	20	—	—	52	22	8.00-8.42	—	Popov (1932)†
		—	—	—		—	—	—	—	—	20-80	—	—	—	Scheunert & Krzywanek (1930)
Rumen liquor		—	—	—		—	—	—	—	—	—	—	188	—	Watson (1933)
		—	—	—		—	—	—	—	—	—	—	—	—	Weyers (1937)
		1.8	0.94	—		—	—	—	26	—	87	50	—	—	Brunnuch & Winks (1931)
Goat	Parotid	0.71-1.96	0.02-1.02	—		10-40	—	—	—	—	—	10-12	—	—	Trautmann & Albrecht (1931)
		—	—	—		—	—	—	—	—	—	—	—	8.12-8.32	Popov (1932)†
Ox	Mixed	1.25-1.67	0.83-0.86	0.6		—	—	—	—	—	—	—	205	—	Markoff (1913)
Cattle	Mixed	—	—	—		—	—	—	—	—	—	—	—	8.1	Schwarz & Hermann (1924)
		0.42-2.2	—	—		—	—	—	—	—	—	—	—	8.8	Chiraszcz & Schechtelowna (1930)
Buffalo	Parotid	0.85	0.08	—		Trace	277	—	—	0	350	15	—	8.8	Sharma (1936)

* To facilitate comparison, some data have been expressed in a form different from the original

† Quoted by Bablan (1944)

cow by Markoff (1913), the diet being hay in each case. The analyses of the saliva of various ruminants reported in the literature are given in Table 2. Zuntz (1913) claimed that the amount of alkali secreted each day in the saliva of the ox must be about six times that contained in the blood and Markoff (1913) calculated that his cow produced 300-350 g alkali expressed as sodium bicarbonate. The latter figure may be compared with the value of 320 g volatile acid (calculated as acetic acid) in the rumen and reticulum of an ox, reported by Elsdon, Hitchcock, Marshall & Phillipson (1946). It is evident that in this species the saliva flowing into the rumen each day supplies a large volume of liquid for the suspension of the ingesta and a large amount of alkali for the neutralization of acids produced in the rumen.

The analyses in Table 2 are rather incomplete, often being limited to the dry matter and titratable alkalinity. In view of the importance of the saliva to the ruminant, a more detailed knowledge of its composition seemed to be required.

In the present work a study has been made of both the mixed and parotid secretions of sheep. First, the composition was investigated by analyzing samples for dry matter, ash and various constituents, particularly inorganic ions, which might be supplied from the blood of the animal. Secondly, special attention was paid to the determination of the carbon dioxide content and the hydrogen ion concentration. During the investigations a number of observations was also made on the rate of secretion and the daily output of saliva.

METHODS

Collection of samples. Mixed saliva was obtained either as it dripped from the tongue of a sheep under chloralose or Nembutal (pentobarbital) anaesthesia, or from a normal sheep by means of a sponge gag, Pl 1 a and b, devised for the purpose. The samples were usually uncontaminated, though occasionally they appeared to contain small amounts of regurgitated rumen contents.

Parotid saliva was obtained by cannulating the duct from the parotid gland under Nembutal anaesthesia and leading the saliva off through valve rubber tubing, Pl 1 c. Collection of samples was commenced about 1 hr after the effect of the anaesthetic had ceased and a portion was taken under paraffin oil to prevent loss of carbon dioxide for the estimation of the pH and carbon dioxide content.

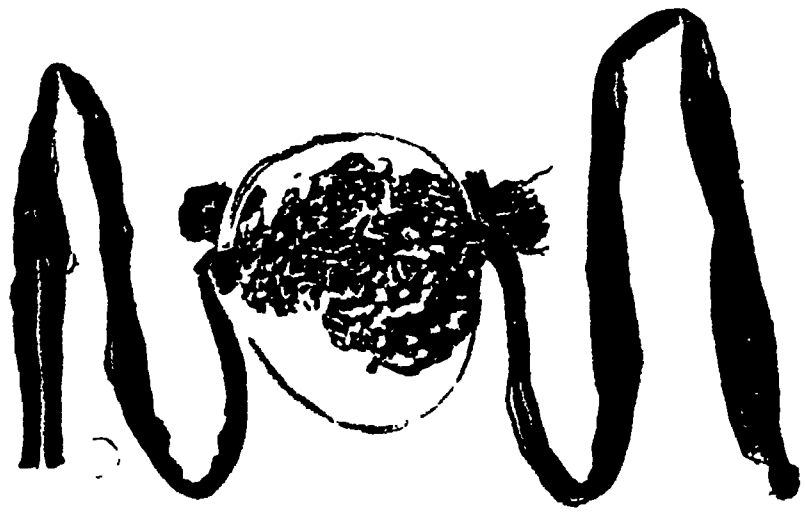
Rumen liquor was obtained either from a sheep with a rumen fistula or from freshly killed animals. Care was taken to avoid undue exposure to air by covering with a layer of liquid paraffin, and bacterial activity was arrested by the addition of merthiolate (sodium ethylmercurithiosalicylate).

Chemical methods. The dry matter and ash were determined by pipetting 5 ml of saliva into a small tared crucible, evaporating on a water bath and drying in a hot-air oven at 110° to constant weight, finally it was ashed and weighed again.



(a) Mixed saliva

(c) Parotid saliva



(b) Sponge gag

Nitrogen was determined by the micro Kjeldahl method, reducing substances by Maclean's method for blood sugar, the carbon dioxide content with Van Slyke's volumetric apparatus, and the hydrogen ion concentration potentiometrically using a quinhydrone electrode.

Inorganic ions were estimated by standard methods for blood serum as follows: sodium, Kramer & Gittleman (1924), potassium, Kramer & Tisdall (1921), calcium, Clark & Collip (1925), magnesium, Denis (1922), phosphorus, Fiske & Sabbarow (1925), chloride, at first by the method of Whitehorn (1921) and later by the method of Sendroy (1937).

RESULTS

Detailed analyses of saliva

Mixed saliva Eleven samples were obtained from four anaesthetized and one normal sheep. The particulars of the samples and the results of the analyses are given in Table 3.

The dry matter was 1.0–1.39 and the ash 0.73–0.9 g/100 ml, the organic matter was thus quite low. A small amount of nitrogen was found but its nature was not determined. No reducing sugars were detected. The chief constituents were inorganic sodium and bicarbonate predominated, potassium, chloride and phosphate were present in smaller amounts, and calcium and magnesium were found in traces.

As the samples were not very uniform in origin they call for individual comment. With sheep 2, sample A was obtained early, and B late, in the period of anaesthesia, the analyses show, however, that a fairly steady composition was being maintained. Of the samples from sheep 3 and 4, sample a appeared to contain a small amount of regurgitated rumen contents, whereas sample b was uncontaminated, it is evident that the effect of this contamination was negligible.

Four samples were obtained from sheep 5 at intervals during 24 hr by means of the sponge gag. Some constituents, notably the phosphorus and carbon dioxide, showed considerable variation during this period.

The differences in the composition of saliva from different animals were more marked for certain constituents, particularly potassium, phosphorus and carbon dioxide, than for others. These differences, however, cannot be understood without an adequate knowledge of the composition of the individual salivary secretions. Of the latter, that from the parotid gland is the most important and has therefore received special attention in this investigation.

Parotid saliva Samples were obtained at intervals throughout the day from six sheep with parotid fistulae. Of these sheep, no. 2 and three others were wethers, no. 4 an approximately 6 months old lamb and one an aged ram. For four of the animals the experiments lasted until the third day after cannulation by which time the rate of production of saliva

had become inconveniently slow. In two animals the experiments were stopped on the 1st and 2nd days after cannulation respectively as the secretion had dried up and become almost negligible. At the end of the experiment one sheep had an infection of the duct but not the reduction in size and structural alteration in the gland reported by Scheumert & Trautmann (1921) to occur some time after establishment of a parotid fistula. In two other animals examined there was no apparent reason for the stoppage of secretion, in fact, the gland subsequently healed up and was observed to function normally again.

Of the results obtained, those for two sheep only are given in Table 4 because of limitations of space. In addition to these analyses, a number of samples was examined qualitatively by the biuret, ferric chloride and Benedict tests for protein, thiocyanate and reducing sugars but all these tests proved negative.

The results for sheep 2 showed lower values for carbon dioxide content and pH than those for the other sheep and are considered atypical though the reason for their being so is not apparent. The results for sheep 4 were typical of those obtained for the four remaining sheep. Only those samples collected on the first 2 days of the experiments on these five animals have been considered as normal. Those obtained on the 3rd day were excluded in taking average values because by this time the secretion was drying up as already noted and also changes in its composition had often become evident. On the whole these results showed that the composition of the parotid saliva was not affected by the age of the animals, that it was similar to that of the mixed saliva quoted in Table 3, and hence that the latter secretion was largely composed of parotid saliva.

The results for the cations do not call for much comment but those for the anions showed the following features. The phosphorus (inorganic), average 81 mg/100 ml, showed extensive variations, both between one animal and another and among samples from the same animal. The values tended to decrease slightly when the animal was eating or ruminating and to fall gradually during the course of the experiment to a fraction of the original amount. The chloride content, average 61 mg/100 ml, also varied definitely from animal to animal but, on the other hand, it increased considerably during the course of the experiment, in the most extreme case, that of sheep 4, from 23 to 238 mg/100 ml. These two effects were approximately compensatory, as the third chief anion, the carbon dioxide content, was more steady, though in three cases there were indications of lower values setting in on the 3rd day of an experiment. Its value, average 233 ml/100 ml, was approximately equivalent to 0.1N bicarbonate solution and showed an

Table 3 Composition of the mixed saliva of sheep

Sheep no	Sample	Content (g/100 ml)			Content (mg/100 ml)							CO ₂ (ml/100 ml)	pH
		Dry matter	Ash	Reducing com pounds	N	P							
						Na	K	Ca	Mg	(inorg)	Cl		
(a) Anaesthetized sheep													
1	—	1.0	0.8	—	—	428	30	3.0	—	37	25	283	8.6
2	A	—	—	0	—	370	16	1.6	0.8	57	35	207	8.6
	B	—	—	0	9	392	22	2.0	0.7	52	41	212	8.5
3	a	1.2	0.9	0	—	399	29	2.3	1.0	72	34	221	8.4
	b	—	—	—	19	414	30	—	0.9	83	—	200	—
4	a	1.0	0.9	0	—	399	26	2.5	0.8	38	43	255	8.6
	b	1.0	0.9	0	20	387	—	2.4	0.6	—	43	252	—
(b) Normal sheep													
5	5 p m	—	—	—	—	429	45	2.3	—	52	—	176	8.6
	9 30 a m *	1.10	0.73	—	—	429	45	2.3	—	53	—	117	8.7
	12 30 p m	1.39	0.90	—	—	402	46	2.1	—	80	—	152	8.6
	4 30 p m	1.39	0.89	—	—	402	40	2.4	—	62	—	172	8.5

Treatment sheep 1, chloralose anaesthesia, sheep 2-4, Nembutal Samples A and B collected early and late in period, under anaesthetic
amination suspected with regurgitated rumen contents, sample b no contamination evident

* Before feeding

Treatment sheep 1, chloroform anaesthesia, sheep 2-4, Nembutal. Samples A and B collected early and late in period, under anaesthetic. Sample a slight contamination suspected with regurgitated rumen contents, sample b no contamination evident.

* Before feeding

Table 4 Composition of the parotid saliva of sheep

Sheep no	Day of exp	Sample		Content (g/100 ml)			Content (mg/100 ml)							Time to secrete 10 ml samples		
		Time	Dry matter	Ash	N	Na	K	Ca	Mg	P	Cl	CO ₂ (ml/100 ml)	pH	(a) min sec	(b) min sec	(c) min sec
2	1	12 30 p m	1.38	1.15	20	441	39	1.5	1.0	106	77	106	7.69	—	—	—
		4 30 p m	—	—	—	447	45	1.3	1.1	120	—	118	7.79	—	—	—
		7 30 p m	1.28	1.10	16	425	35	2.0	1.0	129	89	146	—	—	—	—
4	1	12 noon	1.30	1.00	17	410	12	0.4	0.5	92	23	246	8.18	10	0	9
		1 p m *	1.25	0.95	21	396	14	1.0	1.3	64	19	267	8.20	2	0	3
		4 30 p m	1.30	1.05	23	424	19	0.3	0.5	102	19	206	8.23	6	0	7
		11 p m	1.35	1.15	24	439	18	0.2	0.5	125	20	249	8.17	10	30	8
2		5 30 a m	1.30	1.10	28	412	22	0.7	0.7	82	51	285	8.06	8	0	7
		10 a m	1.20	0.90	25	394	19	0.7	0.5	59	80	245	8.20	6	30	7
		10 30 a m *	1.15	0.85	23	387	25	0.6	0.4	48	94	250	8.23	2	45	3
		5 p m	—	—	31	391	22	0.1	0.8	57	88	207	8.04	13	0	21
		11 p m	1.25	0.90	35	395	22	0.5	0.9	79	99	202	8.03	30	0	26
3		6 a m †	1.10	0.80	29	352	26	0.6	1.1	28	174	205	8.15	4	0	4
		10 a m	—	—	31	353	15	0.6	0.9	28	148	201	8.12	18	0	27
		11 a m *	1.10	0.90	25	357	14	0.6	0.5	19	180	191	8.08	12	0	7
		3 40 p m †	—	—	29	370	13	0.8	0.5	20	238	172	7.97	—	10	30

Sheep 1, 3, 5 and 6 gave similar results to sheep 4

* Feeding

† Rumination

‡ After injection of pilocarpine

Table 3, and probably most of those hitherto reported in the literature for ruminant saliva, must be regarded as indicating too low a carbon dioxide content and thus too alkaline a reaction

The variation in the carbon dioxide content at different times of the day was often as great as 100% but was not regular enough to suggest any correlation with the time of feeding which took place after the first sample had been taken. Such a correlation might become apparent if the observations were repeated with more detailed information available about the feeding and ruminating habits of the animals. As the variation was greater than that reported in Table 4 for parotid saliva, it was probably due to changes in the proportions, rather than the composition, of the individual secretions.

The carbon dioxide content of these samples was on the whole lower than was recorded for the anaesthetized sheep in Table 3, the values after equilibration showed a range of 25–200 ml/100 ml. The lowest figures were mainly due to sheep 1 and 2,* but it is not possible to say that these were definitely abnormal owing to the small number of animals which have been studied so far.

saliva is not drastically reduced on entering the rumen and is ample for its function as a buffer.

The carbon dioxide content and pH of parotid saliva
The number of sheep whose parotid saliva could be studied in such detail as reported in Table 4 was limited, but it was possible to obtain single samples sufficient for the determination of the carbon dioxide content and of the pH from seven other sheep which were being used for acute experiments under spinal anaesthesia, and thus to obtain more representative figures for these values. The results are given in the second and third columns of Table 6. The carbon dioxide content of these samples was fairly uniform (average 237 ml/100 ml) and of the same order as reported for sheep 4, Table 4. The average pH was 8.23.

*Rate of secretion and daily output of
parotid saliva*

During the experiments on sheep with parotid cannulae, frequent observations were made on the rate of secretion of the saliva. Some of the data are included in Table 4 and some additional results are given in Table 7. Both eating and rumination

Table 6 *The CO₂ content and the pH of pure parotid saliva of sheep*

Sample from ewe anaesthetized on	CO ₂ (ml./100 ml)	pH		
		Found, at room temperature	Calculated, for	
			15°	38°
24 i 44	277	8.30	8.26	8.16
26 i 44	241	8.46	8.20	8.10
29 i 44	253	8.23	8.22	8.12
2 ii 44	227	8.05	8.17	8.07
29 ii 44	176	8.11	8.06	7.96
1 iii 44	254	8.14	8.22	8.12
3 iii 44	232	8.35	8.18	8.08
Average	237	8.23	8.19	8.09

On flowing into the rumen, the mixed saliva becomes diluted with the food and water of the diet and partly neutralized by the acids produced by fermentation in the rumen. The carbon dioxide content of the rumen liquor may therefore be expected to show considerable variation in relation to the diet of the animal and the time since feeding. However, a few samples of rumen liquor were analyzed for comparison with the mixed saliva. The results showed values of 40–170 ml/100 ml from which it appears that the bicarbonate content of the mixed

brought about a considerable increase in the rate of secretion. The mere sight of food, on the other hand, caused no psychological stimulation. The effect of pilocarpine was not apparent until 30 mg had been given intravenously. When 5 grains Nembutal was given intravenously, this was sufficient to cause the sheep to stagger on its feet, but appeared to have only a transient effect on the rate of secretion.

It was possible in these experiments to collect the saliva from the cannulated parotid of some of the sheep for considerable periods and thus to obtain a reliable estimate of the daily output. The results are summarized in Table 8. The volume from one gland was 930–1840 (average 1310) ml/24 hr. At slaughter of sheep 6, Table 8, the cannulated parotid gland was found to weigh 35 g. As it secreted 930 ml in 24 hr, its activity can be expressed as 27 ml/24 hr/g of tissue.

* Sheep 2, Table 4 and sheep 1 and 2, Table 5, had well established rumen fistulas. As the carbon dioxide content of the saliva from these animals was particularly low, one wonders whether the rumen fistulas had made them abnormal in this respect. Sheep 6, Table 4, however, possessed a rumen fistula for years but it had become closed at the time of the salivary studies, the saliva of this sheep had a normal carbon dioxide content.

Table 7 Observations on the rate of parotid secretion in the sheep

Sheep		Time to secrete 10 ml samples			
		(a)		(b)	
		min	sec	min	sec
4	Idle	8	0	7	0
	Ruminating	2	30	2	30
	Idle	8	30	6	0
	Ruminating	2	15	2	30
	Idle	10	30	—	—
	Ruminating	4	0	4	30
5	Idle	11	12	—	—
	Ruminating	4	38	4	44
4	Before injection	43	0	—	—
	After 2 mg pilocarpine subcutaneously	41	0	—	—
	After 30 mg pilocarpine subcutaneously	32	0	—	—
	After 30 mg pilocarpine intravenously	7	0	10	30
	Before injection (5, 2, 3, 3 drops/min)	—	—	—	—
	After 5 grains Nembutal intravenously (14, 7, 3, 5, 4, 4, 7, 6 drops/min)	—	—	—	—

Table 8 Volume of saliva collected from a parotid gland of sheep

Sheep no	ml./24 hr
1	1100
3	1440
4	1840
5	1240
6	930
Average	1310

DISCUSSION

Mixed saliva

Comparison with human saliva The special importance of saliva to the ruminant was emphasized in the introduction. It is therefore relevant to compare the present results for sheep with those for an animal with a simple stomach. To this end, figures quoted for the composition of mixed human saliva in a number of text books are presented in Table 9, together with the range of the data given in Table 3. The saliva of sheep has less dry matter and calcium, a higher ash, sodium, carbon dioxide content and pH and about the same level of magnesium and chloride, as compared with human saliva. The potassium and chloride are variable in both.

Relation to function These differences in composition are clearly related to the special role of sheep's saliva. The large amount of carbon dioxide as bicarbonate together with the high phosphate content are functionally most important for rendering the fluid an ideal buffer for the bacterial digestion that goes on in the rumen. The bicarbonate secreted each day by the parotids can be calculated from the present data to be on the average 2.73 equiv. This may be compared with a total of 63.6 g (1.05 equiv.) of volatile acids, as acetic acid, found in the rumen of sheep by Elsdon *et al* (1946). The salivary phosphate may have a function in addition to that of a buffer, as it was suggested by Watson (1933) that it might be important in maintaining the bacterial population of the rumen. This may well be the case as van der Wath (1942) showed that the number of these organisms was stimulated by a supplement of phosphate to a diet low in this constituent.

Parotid saliva

Typical composition The results obtained in the experiments on sheep with cannulated parotid ducts (see Table 4) have been averaged to give a typical composition for the sheep's parotid saliva shown in Table 10. In arriving at this, the data obtained on

Table 9 Comparison of the composition of mixed saliva of sheep and of man

Animal	Literature reference	Content (g/100 ml)		Content (mg/100 ml.)						CO ₂ (ml/100 ml.)	pH
		Dry matter	Ash	Na	K	Ca	Mg	P	Cl		
Sheep	—	1.0-1.4	0.7-0.9	370-462	16-46	1.6-3.0	0.6-1.0	37-72	25-43	117-283	8.4-8.7
Man	(Thorpe 1938)	0.3-1.4	0.2	26	38	8	1	10	50	—	—
Man	(Shohl 1939)	—	0.219	20	100	6	2	18	40	22-33	6.6
Man	(Fearon 1946)	0.6	—	20-30	30-100	8	1	3-19	40-50	—	6.35-6.85

the 3rd day of the experiments and all those for sheep 2 were excluded for reasons already stated (p 101) The composition is expressed as mg /100 ml and m equiv /l In calculating the latter, it has been assumed that at the pH of the saliva, the carbon dioxide exists as HCO_3^- and the phosphorus as HPO_4^-

total carbon dioxide content and the partial pressure of the carbon dioxide, provided the composition of the solution is known The hydrogen ion concentrations of the samples of saliva in Table 6 were therefore calculated from the carbon dioxide content (1) at room temperature for comparison with the values actually found and (2) at body temperature

Table 10 *Average composition of the parotid saliva of sheep*

Content (g /100 ml)		Content (mg /100 ml)							CO_2 (ml / 100 ml)
Dry matter	Ash	N	Na	K	Ca	Mg	P	Cl	
1.28	0.97	20	408	32	0.8	0.8	81	61	233
		Content (m equiv /l *)							
		177	8	0.4	0.6	52	17	104	

* P assumed to exist as HPO_4^- , CO_2 assumed to exist as HCO_3^- Total cations, 186, total anions, 173 m equiv

Completeness of the analyses The amounts of the various ions given in Table 10 can be calculated to be equivalent to a dry matter content of 1.29 g /100 ml This figure is in good agreement with the average dry matter actually found, viz 1.28 g /100 ml, and indicates that no major constituent had been overlooked On the other hand, when the results are expressed as m equiv, it is apparent that the total cations, 186 m equiv /l, exceed the total anions, 173 m equiv /l This difference might be accounted for as sulphate which exists in small amounts in the blood or as volatile acids which were shown by Barcroft, McAnally & Philipson (1944) to occur in the blood of the ruminant, though the amount found in arterial blood was very small Another possibility might be the presence of an organic acid, such as lactic acid, derived from the metabolism of the gland itself The present results, however, are sufficient to enable one to suggest a formula for synthetic saliva which may prove useful to biochemists and bacteriologists wishing to simulate the conditions in the rumen of the sheep in the study of ruminant digestion Such a formula is given in Table 11, and is based on the data in Table 10

Table 11 *Suggested composition for synthetic saliva*

Salt	mmol /l	g /l
NaHCO_3	117	9.8
$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	26	9.3
NaCl	8	0.47
KCl	8	0.57
CaCl_2 anhyd	0.2	0.04
MgCl_2 anhyd	0.3	0.06

Hydrogen ion concentration The saliva was found by potentiometric determinations to have a pH of c 8.23 (Table 6), due to the large amount of bicarbonate present The hydrogen ion concentration may be calculated indirectly in such a solution from the

to give the reaction of the saliva as secreted The steps in the calculations, the assumptions made and the values taken for the various constants are given below

An approximate value of the ionic strength (μ) of the saliva was calculated, assuming the typical composition given in Table 10, whence

$$\mu = \frac{1}{2} \sum m_i z_i^2 = 0.18,$$

where m_i = molar concentration of an individual ion and z_i its valency

The first apparent stoichiometric dissociation constant of carbonic acid at this ionic strength was calculated from the thermodynamic ionization constant The simplified Debye Hückel equation for uni-univalent electrolytes in dilute solutions gives the relation of these three quantities as

$$\text{pK}' = \text{pK} - A\sqrt{\mu},$$

in which A is a constant for a given solvent This equation was found by Hastings & Sendroy (1925) to hold for carbonic acid in solutions containing sodium bicarbonate and chloride As the predominating ionic species in saliva are sodium and bicarbonate and as Bray (1925) found that the activity of the bicarbonate ion is different in solutions of potassium as compared with sodium salts, the above equation was employed in the present calculations, rather than any more recent but empirical equation based on measurements made with potassium salts Values for pK'_1 at the two required temperatures were taken from Shedlovsky & MacInnes (1935) and for A from MacInnes (1939) Then for carbonic acid in saliva

$$\begin{aligned} \text{at } 18^\circ, \quad \text{pK}'_1 &= 6.43 - 0.50\sqrt{0.18} = 6.22, \\ \text{at } 38^\circ, \quad \text{pK}'_1 &= 6.32 - 0.52\sqrt{0.18} = 6.10 \end{aligned}$$

Before the pH of the saliva can be calculated it is necessary to know the value $[\text{H}_2\text{CO}_3]$, representing the concentration of unionized carbonic acid and physically dissolved carbon dioxide, a value depending on the carbon dioxide tension As no data could be found in the literature for the carbon dioxide tension of sheep's saliva, blood or alveolar air, the value for human arterial blood was taken, giving

$$[\text{H}_2\text{CO}_3] = 2.4 \text{ ml./100 ml}$$

This seemed justified for two reasons (1) It has been shown that equilibration with human alveolar air restored the original carbon dioxide content to samples of sheep's saliva which had lost carbon dioxide through exposure to air. The differences in the partial pressure of carbon dioxide in the two species cannot therefore be very great. (2) Small differences between the assumed and actual values of $[H_2CO_3]$ will have only a small effect on the calculated pH values.

The pH of the samples of saliva were calculated from their carbon dioxide content given in Table 6, employing the Henderson Hasselbach equation, viz

$$pH = pK' + \log [Salt]/[Acid]$$

This becomes, when applied to the sheep's parotid saliva

$$\text{at } 15^\circ, \quad pH = 6.22 + \log [Total CO_2 - 2.4]/[2.4],$$

$$\text{at } 38^\circ, \quad pH = 6.10 + \log [Total CO_2 - 2.4]/[2.4]$$

The calculated pH values at 15 and 38° are given in Table 6 together with the experimental data on these samples. It will be seen that the calculated values at 15° are in good agreement with those found potentiometrically at room temperature, the averages being 8.19 and 8.23, respectively. The calculated values at 38° show that the saliva is secreted at a pH of about 8.1. This value is less alkaline than many reported for ruminant saliva but is considered more accurate.

Calcium. The saliva of the sheep contains much less calcium than the blood. This is probably because in the absence of significant amounts of protein the calcium must be largely ionized and at the pH of the saliva only small amounts of calcium ions can be in equilibrium with the carbonate and phosphate present. The maximum ionized calcium possible at body temperature was calculated for a solution with the composition of sheep's parotid saliva, as follows.

The concentration of the carbonate ion was obtained from an equation derived from the negative logarithmic expressions for the first and second stages of ionization of carbonic acid

$$pK_1' = pH + p[HCO_3^-] - p[H_2CO_3], \quad (i)$$

$$pK_2' = pH + p[CO_3^{--}] - p[HCO_3^-] \quad (ii)$$

Adding (i) and (ii) and rearranging

$$p[CO_3^{--}] = pK_1' + pK_2' - 2pH + p[H_2CO_3] \quad (iii)$$

The values $pK_1' = 6.10$ at 38° and $\mu = 0.18$ have already been calculated. The value of pK_2' under these conditions was obtained from the equation of Hastings & Sendroy (1925)

$$pK_2' = 10.22 - 1.1\sqrt{\mu} = 9.75$$

The pH was taken as 8.1 and the apparent concentration of unionized carbonic acid again assumed to be 2.4 ml/100 ml. The latter expressed as molar quantities gives

$$[H_2CO_3] = 1.07 \times 10^{-3},$$

and therefore $p[H_2CO_3] = 2.97$. Substituting these values in equation (iii) gives

$$p[CO_3^{--}] = 6.10 + 9.75 - 2 \times 8.1 + 2.97 = 2.62$$

The equilibrium concentration of calcium ions corresponding to this amount of carbonate was calculated from

the solubility product of calcium carbonate. The value of the latter at 38° was taken from Sendroy & Hastings (1927a)

$$pK'_{s.p.} = 8.58 - \frac{4.94\sqrt{\mu}}{1 + 1.85\sqrt{\mu}} = 7.40 \text{ at } \mu = 0.18$$

$$\begin{aligned} \text{Then } p[Ca^{++}] &= pK'_{s.p.} - p[CO_3^{--}] \\ &= 7.40 - 2.62 = 4.78 \end{aligned}$$

$$\begin{aligned} \text{Whence } [Ca^{++}] &= 1.7 \times 10^{-5} \\ &= 0.07 \text{ mg/100 ml} \end{aligned}$$

A series of similar calculations was made for tertiary calcium phosphate, employing the values for the three dissociation constants of phosphoric acid and the solubility product of the calcium salt, given by Sendroy & Hastings (1927a, b). The equilibrium concentration of the calcium ion with the phosphate concentration found in saliva was found to be $[Ca^{++}] = 0.1 \text{ mg/100 ml}$.

It follows from the above considerations that the low salivary calcium is secondary to the high bicarbonate and phosphate contents. The values actually found are higher than the calculated figures. One can only speculate whether the discrepancy is due to experimental error in determining the small amount present, to the existence of colloidal calcium phosphate, to supersaturation or to an undissociated complex.

Comparison with sheep's serum. As the dry matter of the parotid saliva of sheep is largely mineral and the mineral constituents of such secretions must be derived from the blood, it is interesting to see how the mineral compositions of these two fluids compare. The average composition of the parotid saliva has therefore been presented in Table 12 together with figures for the composition of sheep's serum.

Table 12 Comparison of the composition of parotid saliva and serum of sheep

Constituent	Parotid saliva	Serum
Dry matter (g/100 ml.)	128	83
Nitrogen (mg/100 ml.)	20	865
Reducing sugars (mg/100 ml.)	Absent	105
Na (mg/100 ml.)	408	350-380*
K (mg/100 ml.)	32	30-40*
Ca (mg/100 ml.)	0.8	10-11
Mg (mg/100 ml.)	0.8	2-5
P (inorganic) (mg/100 ml.)	81	45-6*
Cl (mg/100 ml.)	61	370
CO ₂ (ml/100 ml.)	233	56

* From Shearer & Stewart (1931), remainder from Dukes (1943)

The concentrations of the principal cations, sodium and potassium, are similar in both fluids. The concentrations of both calcium and magnesium are less in the saliva. The greatest differences, however, are shown by the anions. The carbon dioxide content of the saliva is about four times the normal alkali reserve of sheep's blood. It might be expected that the continuous loss of so much alkali from the

blood as bicarbonate would tax the ability of the sheep to maintain the acid-base balance in the blood. This loss, however, may be offset not only by reabsorption lower down in the digestive tract but also by the abomasal secretion which in the sheep is not only acid but also continuous. The phosphorus of saliva is entirely inorganic, variable in amount and has a concentration about fifteen times that of the serum inorganic phosphorus. This is in agreement with the data of Watson (1933) who found in addition that the phosphorus in the saliva appeared to depend on the inorganic phosphorus in the serum. The chloride occurs to about one sixth of the extent of its normal level in the serum.

Nature of the secretion It is evident from Table 12 that the parotid gland mainly secretes ionized salts. This suggests that the difference between the ionic composition of serum and saliva might be due to a Donnan effect of the serum proteins. However, it is evident by inspection of the data that the required relation for uni-univalent electrolytes, namely,

$$[\text{Base}] [\text{Acid}]_{\text{saliva}} = [\text{Base}] [\text{Acid}]_{\text{serum}}$$

does not hold. Hence saliva cannot be regarded as a transudate in equilibrium with the blood.

If the two fluids are in static equilibrium, the general condition must hold that there is no net gain or loss of free energy involved in the formation of the saliva. Assuming that all the constituents of the latter are derived from the serum, the associated change in free energy can be calculated without making any assumptions as to the mechanism of saliva formation.

The decrease in free energy, $-\Delta F$, for 1 l. of saliva is given by the expression

$$-\Delta F = \sum nRT \log_e (a_{\text{serum}}/a_{\text{saliva}}),$$

where n is the number of mol. of a constituent transferred from a very large volume of serum to 1 l. of saliva and a its activity. The ratios of the activities can be sufficiently approximated by the ratios of the molar concentrations. In calculating the latter, the median value has been employed for those serum constituents for which a range only has been quoted in Table 12. Serum calcium has been assumed to be 60% ionized. Phosphate in the saliva has been regarded as entirely HPO_4^- and the proportion existing in this form in the serum has been calculated for pH 7.4 using the expression

of Hastings & Sendroy (1927a) for pK_2' of phosphoric acid. The work of transference of other phosphate ions has been taken as negligible.

In this way it is estimated that

$$-\Delta F = -130 - 163 = -293 \text{ cal.}$$

the first term refers to the transference of salts and the second to the transference of water. The saliva is therefore not a dialysate in equilibrium with serum as its formation requires a small but definite supply of energy. Whether this can be obtained from the work of ultrafiltration done on the saliva or whether it must be supplied through the metabolism of the gland itself is a problem for further investigation.

SUMMARY

1 The composition of the mixed and parotid salivary secretions of the sheep has been studied.

2 The dry matter, 1.0–1.4 g/100 ml, of both fluids was mainly inorganic but contained small amounts of nitrogen, 9–36 mg/100 ml. The mixed secretion usually appeared to consist largely of parotid saliva. The concentrations of total salts in the latter, 180 m equiv/l., and of the principal cations, sodium and potassium, were similar to those occurring in serum. The concentrations of the anions, of bicarbonate, and of phosphate and chloride, were approximately four times, fifteen times and one-sixth respectively as much as those obtained in the serum.

3 The carbon dioxide content of the parotid saliva, mixed secretion and rumen liquor was found to be 233, 25–200 and 40–170 ml/100 ml, respectively. The parotid saliva had an average pH of 8.2 and was calculated to be actually secreted with a pH of 8.1.

4 The amount of saliva formed by a single parotid gland ranged from 930 to 1840 ml in 24 hr.

I must thank Dr A. T. Phillipson, Unit of Animal Physiology, Cambridge, for cannulating the parotid ducts of the experimental animals and for his continued help and encouragement in this investigation.

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Studies in Vitamin A

6 CONVERSION IN VIVO OF VITAMIN A ALDEHYDE (RETINENE₁) TO VITAMIN A₁

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Retinene₁ is a fairly stable protein free substance first obtained from rhodopsin (visual purple), the photolabile chromoprotein of the dark adapted retina. It has recently been shown to be the aldehyde of vitamin A₁ (Morton & Goodwin, 1944; Ball, Goodwin & Morton, 1948). Chemical methods for its preparation have been described (Hunter & Hawkins, 1944; van Dorp & Arens, 1947), and a very simple method, in which a solution of vitamin A₁ alcohol in light petroleum is left to stand over powdered pyrolusite in the cold (Ball *et al* 1948), has made it easily accessible.

The present paper is concerned with the fate of retinene₁ when presented to body tissues other than the retina. In particular, it was desired to find out whether it possessed *per se* vitamin A₁ activity. The substance, in a preparation containing no detectable vitamin A₁ but some 75% of retinene₁, has been administered orally and parenterally to vitamin A₁-depleted rats. It has been found to be easily convertible to vitamin A₁, particularly in the gut wall

75% of the pure substance, but unchanged vitamin A₁ could not be detected spectrophotometrically. It was stored as a solution in light petroleum at 0° in the dark, under which conditions it was quite stable.

Solution in oil. A known quantity of refined ground nut oil was added to a measured volume of the stock retinene solution in light petroleum. The volatile solvent was removed by warming under reduced pressure. The resulting solution in oil was used for oral administration.

Solution in propylene glycol. A similarly prepared solution in propylene glycol was administered intraperitoneally.

Determination. The amounts of retinene₁ administered were determined by spectroscopic methods using the absorption maxima at 368 mμ (cyclohexane solution) and 604 mμ (SbCl₃ colour test) following Ball *et al* (1948). Similar methods using the ultraviolet maxima at 326 mμ and the visible maximum at 617 mμ (SbCl₃ colour test) were used for vitamin A₁. For the few extracts in which vitamin A₁ and retinene₁ occurred together, the correction procedure of Morton & Stubbs (1948) was applied to the ultraviolet absorption curves so as to determine both constituents.

Animals

Preparation for tests. Twenty five newly weaned rats were depleted of vitamin A₁. Because of a shortage of rice starch, the diet low in vitamin A₁ which was previously used (Glover, Goodwin & Morton 1947a) was modified by introducing boiled potatoes in place of rice starch. The cooked potatoes contained very small amounts of provitamin A (mainly as β carotene). Careful analyses showed that each rat may have received the equivalent of 2-3 i.u./day of provitamin A. The

EXPERIMENTAL

Retinene₁

Preparation and storage. Vitamin A₁ aldehyde was prepared by the method of Ball *et al* (1948). The material used had been purified by chromatography and contained about

diet contained enough provitamin to ward off frank deficiency, but too little to delay depletion of the small liver reserves. After 21–28 days, the rats had become almost stationary in weight, and, when the two largest animals were killed, the lipids extracted from livers and intestines gave negative Carr Price tests for vitamin A_1 .

Administration of retinene₁ (a) *Oral* Retinene₁ (1–8 mg) in oil was given either by drops from a calibrated syringe into the back of the mouth, or through a fine rubber catheter, internal vol 0.2 ml., direct into the stomach. For the more quantitative work exactly 1 ml. of oil solution was placed in a small flask, and c. 0.7 ml. was withdrawn into a 1 ml. syringe, which was then at once connected to the rubber catheter which had previously been inserted into the rat's stomach. As much as possible of the oil was expelled from the syringe, and the catheter was then carefully withdrawn while still connected to the syringe. The syringe and catheter were then washed with ether and the rinsings added to the 0.3 ml. in the flask. The retinene in the flask was then determined and the amount given to the animal calculated by difference. The method was convenient for administering c. 0.5 ml. of oily solution. The dose of retinene₁ was varied by adjusting the concentration of solute rather than the volume of solution. Groups of 3–5 animals were used at each dose level, and the individuals were killed at definite intervals of time after dosing.

(b) *Parenteral* Each rat in two groups of three each was given 1–5 mg. retinene₁ in 0.5 ml. of oil by subcutaneous injection. Two rats were given 4.2 mg. retinene₁ in 0.5 ml. propylene glycol by intraperitoneal injection. All eight animals grew normally and showed no ill effects.

Preparation of tissue extracts

The liver, stomach and small intestine were removed from the anaesthetized animal. The stomach and the small intestine were separately flushed with saline, and the contents with the washings were retained for examination. The tissues (e.g. liver) were ground in a mortar with c. 5 times their weight of anhydrous Na_2SO_4 and a little acid washed silver sand, and thoroughly extracted with redistilled ether (Glover *et al.* 1947a). Retinene₁ is unstable in the presence of concentrated alkali, and saponification has therefore to be avoided. In the extraction of macerated and dried intestinal tissue, addition of a little ethanol promotes filtration through the sintered glass funnel (G4), which is otherwise liable to become clogged.

The contents of the stomach and intestines were transferred to a mortar and triturated with ethanol (10–20 ml.). The mixture was then extracted with ether in a separatory funnel.

The lipid extracts were always freed from ether and taken up in a known volume of chloroform or cyclohexane.

RESULTS

Oral administration

In the animals which received the retinene₁ by mouth, the contents of the stomach and the small intestine often contained unabsorbed and unchanged vitamin A_1 aldehyde but no trace of vitamin A_1 . On the other hand, the intestinal wall and the liver con-

tained vitamin A_1 with no trace of retinene₁. These results indicate that the vitamin A_1 aldehyde was reduced to vitamin A_1 alcohol immediately on absorption into the intestinal mucosa. The reduction must have taken place extremely rapidly, as no trace of the retinene was detected in the extract from the intestinal tissue.

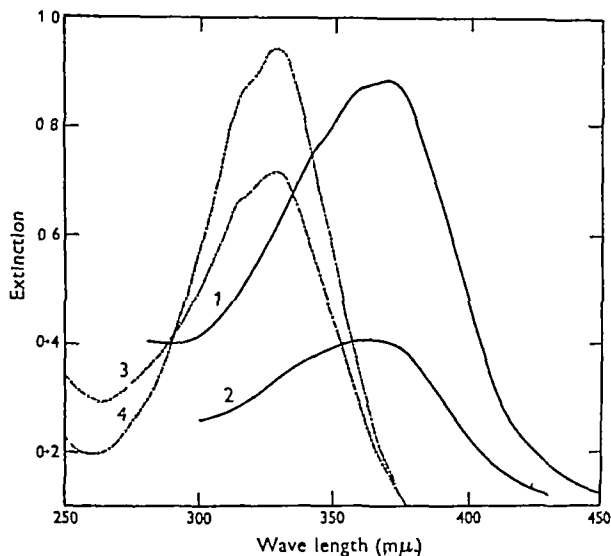


Fig 1. Absorption spectra of lipid extracts (in cyclohexane) of tissues of a rat killed 24 hr. after dosing with 8.0 mg. retinene₁ in 1.0 ml. oil. — Retinene₁ (max. 368 mμ) (1) stomach contents, (2) contents of small intestine — — Vitamin A_1 (max. 328 mμ) (3) gut wall, (4) liver.

Fig 1, which records the absorption spectra of the various extracts in a typical experiment, shows the clear-cut nature of the results. The absorption curves for the liver and small intestine show maxima at 328 mμ, characteristic of vitamin A_1 , whilst those for the contents of the stomach and intestine have maxima at 368 mμ, characteristic of vitamin A_1 aldehyde. Table I summarizes the results obtained in this series of experiments. The excellent recoveries of vitamin A_1 in the animal as shown by the quantitative experiments indicate that retinene₁ is stable in the lumen of the gut and its reduction to vitamin A_1 alcohol *in vivo* must be a very efficient process.

The site of absorption of the retinene₁ into the intestine was briefly investigated. A rat was given a dose of retinene₁ by stomach tube, and was killed after an interval of 15–17 hr. The small intestine was ligatured at the pyloric sphincter and caecum, and was then cut into four parts corresponding as near as could be judged with the duodenum, upper half of jejunum, lower half of jejunum and ileum. Each portion of tissue was examined for vitamin A_1 and the lumen contents checked for the presence of retinene₁. The results are presented in Table 2.

Table 1 *Conversion of retinene₁ into vitamin A₁ in the intestine of the rat*

Experiment	Rat no	Dose of retinene ₁ (μg)	Time between dosing and test (hr)	Retinene ₁ recovered from		Vitamin A ₁ (μg) recovered from		Total recovery of retinene ₁ + vitamin A ₁	
				Stomach (μg)	Contents of small intestine (μg)	Wall of small intestine	Liver	(μg)	Percentage of dose
1*	1}	Nil	Controls	—	—	Nil	Nil	Nil	—
	2}			Nil	Nil	551	1072	1623	41
	3			4000	24	Nil	Nil	1623	41
2*	4	8000	4	4610	91	18	98	4817	60
	5	8000	7.5	4183	4	80	438	4705	59
	6	8000	10	2003	240	141	1619	4063	51
	7	8000	24	799	141	724	2405	4069	51
3*	8	2000	5	152	69	103	518	842	42
	9	2000	10	4	2	60	557	623	31
	10	2000	15	21	0	93	689	803	40
	11	2000	20	1	0	47	725	773	39
	12	2000	25	1	0	43	539	583	29
4†	13	1054	5	821	0	10	64	895	85
	14	1887	8	1080	0	126	545	1751	93
	15	1732	15	0	4	175	1070	1249	72

* Retinene administered orally as drops of oil solution

† Retinene introduced by stomach tube

Table 2 *Distribution of the absorption of retinene₁ along the small intestine of a rat killed 17 hr after receiving 1 mg in oil by stomach tube*

	Retinene ₁ in intestinal contents	Vitamin A ₁ in intestinal wall (μg)
Duodenum	0	6
Jejunum Upper half	0	15
Lower half	0	38
Ileum	Trace	129

It was further of interest to determine if the vitamin A₁ formed in the gut wall became esterified on its way into the blood stream. Light petroleum solutions of the lipid extracts from the intestinal tissue of rats 14 and 15 were examined chromatographically using a bone meal column. In the extract from rat 14 there were 6 parts of esterified vitamin A₁ to 1 part of free vitamin A₁, and in rat 15, 3 parts of ester to 1 part of alcohol.

Parenteral administration

The three vitamin A₁ depleted rats in the first group injected with 4.2 mg of retinene concentrate dissolved in 0.5 ml of refined ground nut oil were killed after intervals of 1 day, 5 days and 5 weeks respectively after dosing. In each case only vitamin A₁ was found in the liver. This suggested, on first thought, that the liver is the locus of conversion of injected retinene₁, but an examination of the site of injection proved otherwise. The surrounding subcutaneous tissues were not examined in detail for

rats 17 and 18, but from their bright yellow colour obviously contained large amounts of unabsorbed retinene₁. The tissue near the point of injection in rat 19 had in 5 weeks become pale yellow. The pigmented portion of tissue was therefore dissected out and extracted. Analysis of the extract by the Carr-Price reaction showed that only vitamin A₁ was present. There was no trace of a reaction product with an absorption band at 664 mμ, i.e. retinene₁ was absent. This observation clearly indicates the presence in the subcutaneous tissue of an enzyme capable of converting vitamin A₁ aldehyde into vitamin A₁ alcohol.

The rats in the second group of three were each given 3.5 mg of retinene₁ concentrate. Two of the animals were killed 3 weeks after dosing and the third 4 weeks later. The subcutaneous tissue and liver were in each case examined as before for residual retinene₁ and for vitamin A₁. The results are summarized in Table 3 and confirm the conclusion already reached. In the case of rat 21, both retinene₁ and vitamin A₁ were found in the subcutaneous tissue. The ultraviolet absorption spectrum of the extract in cyclohexane is recorded in Fig 2 (curve 1). Using the vitamin A correction procedure (Morton & Stubbs, 1946), the true contribution of vitamin A₁ in the extract was calculated (curve 2). Subtracted from the whole (curve 1) this gave the absorption due to retinene₁ plus some irrelevant absorption (curve 3). The co-existence of retinene₁ and vitamin A₁ in the subcutaneous tissue is clear proof that the reduction of the aldehyde to the alcohol can take place there.

Table 3 *Reduction of retinene₁ (vitamin A₁ aldehyde) to vitamin A₁ alcohol in subcutaneous tissue of vitamin A₁-depleted rats, esterification in situ and liver storage*

Experi- ment	Rat no	Dose of retinene ₁ injected in oil (μg)	Time between injection and test (days)	Recovery from subcutaneous tissue				Vitamin A ₁ in liver (μg)
				Retinene ₁ (μg)	Vitamin A ₁ (μg)			
					Total	Ester	Free	
6	17	4200	1}	Observed as deeply stained tissue	—	—	—	54
	18	4200	5}		—	—	—	600
	19	4200	35		0	446	—	—
7	20	3500	21	0	311	214	71	739
	21	3500	21	291	714	631	71	1264
	22	3500	49	0	200	190	200	1175

Portions of the subcutaneous tissue extracts from this series of rats were dissolved in light petroleum, and examined chromatographically using a bone-meal column. A considerable portion of the vitamin A₁ was found in the esterified form. Thus, the subcutaneous tissue fluid contains, in addition to a reductase, an esterase or lipase capable of esterifying

DISCUSSION

In Exp 3, Table 1, where smaller doses of retinene₁ were administered (rats 8–12), the amount of vitamin A₁ in the gut wall diminished rapidly after absorption from the lumen was complete. This fact, together with the failure to detect retinene₁ in either the gut wall or the liver, shows that the retinene₁ is immediately converted to vitamin A₁ on absorption into the gut.

The accumulation of vitamin A₁ in the gut wall during active absorption (rats 4–7, Table 1) shows that entry of retinene₁ into the mucosa, and its conversion to vitamin A₁, are rapid processes compared with the transport of the vitamin away from the gut wall.

The mode of absorption of retinene₁ appears to be very similar to that of vitamin A₁ (Popper & Volk, 1944). Absorption can occur readily along the whole length of the small intestine, but normally does so in the duodenum. In the experiment referred to in Table 2 the retinene₁ in 0.5 ml oil was fed to the fasting animal by stomach tube, and 17 hr after dosing a small amount of unabsorbed fat containing a little retinene₁ had passed well down into the ileum. Under these conditions retinene₁ is apparently very stable in the digestive fluids. This was confirmed by the excellent recovery of vitamin A₁ in rats 13 and 14, Table 1, which received accurately measured doses of retinene₁.

In these quantitative experiments, where retinene₁ was fed by stomach tube, the recovery as vitamin A₁ was exceedingly high (70–90%). This is very much better than the recovery expected after dosing with vitamin A₁ itself. According to Reifman, Hallman & Deuel (1943) the recovery of vitamin A₁ at low dosage levels is only c. 30%. Gray & Cawley (1942) recovered from 40 to 70% of different preparations of vitamin A₁ administered to rats in moderate daily doses (c. 100–500 i.u.). A control experiment was carried out to estimate the recovery of single doses of vitamin A₁ ester given by stomach tube at doses matching those in Exp 4, Table 1. Each of four young rats (not vitamin A₁ depleted but with low

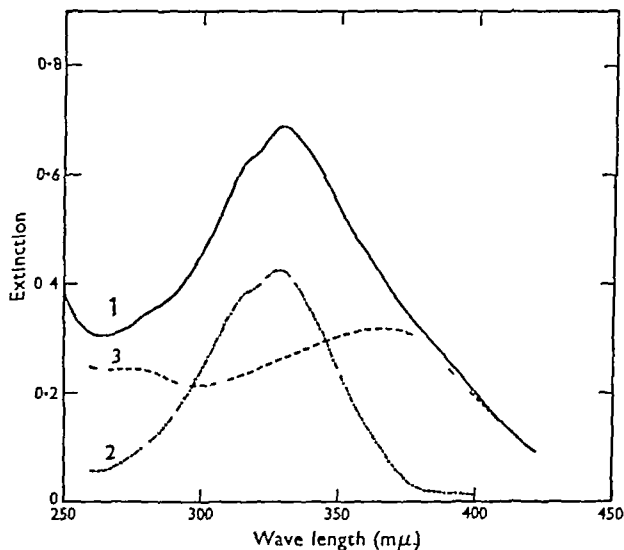


Fig 2 Absorption spectrum (1) of the lipid extract (in cyclohexane) from the subcutaneous tissue of a rat, 21 days after injection with 3.5 mg retinene₁ in 0.5 ml ground nut oil, together with the corrected vitamin A₁ curve (2) and difference curve (3) with maximum at 368 mμ (retinene₁).

free vitamin A₁. This is comparable to the findings of Schönheimer & Yuasa (1929), who had previously noted that free cholesterol injected subcutaneously becomes esterified on absorption. As with the administration of retinene₁ by mouth there is no indication that the liver has any opportunity to effect the reduction.

With intraperitoneal administration the result was much the same. The livers of the two rats, killed 24 and 36 hr after injection, were found to contain 491 and 635 μg of vitamin A₁ respectively. Unabsorbed retinene₁ was observed in the peritoneum

Table 4 Recovery of vitamin A₁ ester administered in single doses by stomach tube

Rat no	Dose of vitamin A ₁ in 0.5 ml oil (μg)	Vitamin A ₁ recovered from		Percentage of dose recovered	
		Intestine (μg)	Liver (μg)	Total	Liver only
23	1513	22	519	35.7	34.3
24	1699	14	267	16.5	15.7
25	1451	20	482	34.6	33.2
26	1686	38	579	36.6	34.3

liver stores, 30 μg/rat) was dosed with c. 1.5 mg of vitamin A₁ ester concentrate in 0.5 ml oil. The recovery of the vitamin from the animals killed 25 hr after dosing ranged from 17 to 37% (Table 4). It is worth noting, therefore, that if large liver stores are a therapeutic objective, retinene₁ is better than vitamin A₁ for attaining that end.

The absorption of retinene₁ from the subcutaneous tissue resembles that from the gut wall, except that it is very much slower. Retinene₁ has been detected 21 days after subcutaneous injection and vitamin A₁ after as long as 49 days. This is not unexpected, as the turn over of tissue fluid (lymph) in a small piece of skin is much slower than in the intestinal wall, which is specially adapted for absorption. The fact that the vitamin A₁ alcohol, formed from the retinene₁ in the skin, underwent esterification before absorption is suggestive that the latter took place mainly via the lymphatic system as from the gut wall. The similarity in the modes of absorption from the skin and the intestine suggest that the enzymes, responsible for the reduction and esterification processes, are widespread in the body tissues.

Bliss (1947) has reported the isolation of a soluble protein from frogs' retinas capable of reducing retinene to vitamin A. Now that tissues other than the retina have been shown to contain the same or a similar enzyme, it may be possible to learn more regarding the nature of the enzyme in the eye. It is probably a reductase, because (a) the excellent recovery of vitamin A₁ from retinene₁ suggests that one molecule of the aldehyde gives rise to one molecule of the alcohol, and (b) it is not likely to be an aldehyde mutase which catalyzes reactions of the Cannizzaro type, producing the alcohol and the acid, since vitamin A₁ aldehyde contains an activated H atom in the α position to the carbonyl group. The fact that the aldehyde is reduced easily to the alcohol, instead of being oxidized to the acid, favours the view that the alcohol grouping is necessary for vitamin A₁ activity.

The oxidation of β-carotene *in vitro* yields a small amount of retinene₁ (Hunter & Hawkins, 1944), which can be reduced to vitamin A₁ in a subsequent stage.

The recent work of Sexton, Mehl & Deuel (1946),

Glover *et al* (1947b), Thompson, Ganguly & Kon (1947), Mattson, Mehl & Deuel (1947), and Wiese, Mehl & Deuel (1947) has shown that the small intestine is the main site of the conversion of β-carotene into vitamin A₁. The earlier work of Goodwin, Dewar & Gregory (1946) also suggests that in the goat the intestine is capable of carrying out the conversion. In the light of all this evidence, together with the present observations, there is no longer any difficulty in accepting the suggestion that the transformation of β-carotene to vitamin A *in vivo* can take place by oxidation of the former to vitamin A₁ aldehyde, which is subsequently reduced to the vitamin A₁ alcohol. Such a process of oxidation and reduction is more probable than a hydrolytic fission.

SUMMARY

1 Retinene₁ (vitamin A₁ aldehyde) administered to rats orally, subcutaneously, or intraperitoneally is converted into vitamin A₁ during absorption. This suggests that the alcohol group (either free or esterified) of vitamin A₁ is essential for its transport and storage.

2 After oral administration the conversion takes place in the gut, and after subcutaneous injection in the subcutaneous tissues. The site of conversion after intraperitoneal injection is uncertain.

3 There is no direct evidence that the liver can convert retinene₁ to vitamin A₁.

4 The conversion represents a simple reduction of the aldehyde.

5 The extracellular spaces of subcutaneous tissue contain an enzyme capable of esterifying vitamin A₁ alcohol.

6 It is suggested that the transformation of β-carotene into vitamin A₁ *in vivo* is more likely achieved by oxidation of the former to retinene₁, which is then rapidly reduced to vitamin A₁, rather than by hydrolytic fission.

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The Composition and Reactivity of Medullated Keratins

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One of the features which distinguishes the coarser wools from the finer merino types is the presence of a medulla. Whereas the finer wools consist only of a cortex and outer scaly structure, the coarser wools contain also a medulla. The chemistry of wool and other keratins has been fairly intensively studied, but, apart from the considerable evidence which suggests a low sulphur content, relatively little is known about the composition of the medulla. Barritt & King (1931), from a comparison of the sulphur contents of medullated and non medullated wools, calculated that the medulla substance contained little, if any, sulphur. Jordan Lloyd & Marriott (1933) found that the medullary cells of goat hair, after separation by treatment with 4N-sodium hydroxide at room temperature, contained only 0.23% sulphur. Bekker & King (1931) showed that the medulla dissected from porcupine quill contained less sulphur than the cortex. The absence of cystine from the medulla was inferred from the appearance of cross sections of the quill stained with sodium plumbite.

Bailey (1937) observed that scoured samples of kemp, which is a heavily medullated fibre, and to a much lesser extent wool, had a low cystine content. One such sample of kemp contained less than 2% of cystine. He concluded that the cystine originally present had been destroyed, probably during the

scouring process. The cystine of kemp would therefore appear to be much more readily attacked by mild alkalis, i.e. the scouring liquor, than that of other keratins.

Stoves (1946 a, b) has recently studied the medulla cells of a number of animal fibres, isolating them from bleached horse hair and other keratin fibres by chemical treatment. Observation of the change in appearance of the cells when treated with a 1% tannic acid solution, 0.1% sodium oleate or 1% saponin suggested that the surface of the medulla cells contained a lipoprotein cholesterol combination. Stoves further showed that when the medulla cells were refluxed with ethanolic sodium hydroxide the solution gave a positive reaction for phosphate, whilst a chloroform extract of the solution gave a positive reaction for sterols.

In the present paper the sulphur analyses of wool and kemp which had been scoured in the laboratory, and wool which had been scoured on a commercial scale, are compared with those of the same wool which had been degreased with solvents. The results of phosphorus determinations on a number of medullated and non medullated keratins, and the examination of hydrolysates of medulla by two dimensional partition chromatography are also given.

METHODS

Materials All the keratins were first extracted with benzene, then with ethanol in a Soxhlet, repeatedly rinsed in water and dried in air. The medulla cells of wool fibres were isolated by the technique of Jordan Lloyd & Marriott (1933), those of feather rachis and porcupine quill by dissection. Kemp fibres were separated from wool fibres by hand.

Analyses Total sulphur estimations on keratins were carried out by the Benedict-Denis method (Barritt, 1934), disulphide S and thiol S estimations by Shimohara's (1935, 1936) methods, methionine by Baernstein's (1936) volatile iodide method, phosphorus estimations by Kuttner & Lichtenstein's (1930) method (see Gortner, 1945; Burmaster, 1946). Analytical results are given as a percentage of the dry weight of the keratin. Qualitative amino acid examination of the keratins was carried out by two-dimensional partition chromatography on paper (Consden, Gordon & Martin, 1944). The keratins were hydrolyzed for 24 hr with 5N HCl, excess acid being finally removed by distillation *in vacuo*. The chromatograms were run in collidine for 2 days and then in phenol ammonia for 1 day.

Scouring methods Part of a Radnor fleece, containing a large proportion of kemp, and a sample of 64's Cape wool were scoured in the laboratory, the tips of the Cape wool fibres being cut off as a preliminary step. The samples were treated with a solution containing 0.2% soap and 0.1% Na₂CO₃ for 3 min at 47°. After passing through rollers, the wool was again treated with 0.1% soap solution for 3 min at 47°, again passed through rollers and air dried. Samples were also taken of wool at various stages during its passage through a commercial scouring set. A 70's quality Australian greasy wool was scoured in a five bowl set. In the first bowl, the wool was given a water steep. The second bowl contained Na₂CO₃, the third soap and Na₂CO₃, the fourth soap, the final rinse being carried out in the fifth bowl. Hourly additions of soap were made to the third and fourth bowls, and the liquors were blown back at the end of a run in the usual way. The temperature of each bowl was taken during the run, and samples of the liquor removed for subsequent pH determination with the glass electrode. Samples of wool were removed before scouring, as it emerged from each bowl and after passage through the dryer. The samples were removed shortly after the start of a run, so that the liquor in the bowls was probably in the most alkaline condition and the grease content low. Before analysis, they were extracted with light petroleum, then with ethanol, rinsed in water and dried.

RESULTS

The phosphorus content of keratins

Table 1 gives the phosphorus content of a number of medullated and non medullated keratins. None of them contains more than a very small amount of phosphorus, which is possibly derived from small amounts of suint which have not been removed by the purification procedure employed, and shown by Freney (1934) to contain phosphates. Some support for this view is given by the fact that non medullated wools may contain as much phosphorus as medullated wools and keratins.

Table 1 *The phosphorus content of keratins*

Keratin	Phosphorus (% of dry wt.)
Blackface wool	0.020
Welsh mountain wool	0.015
Deccan wool (Poona)	0.021
Horsehair (Argentine black mane hair)	0.039
Kemp from Radnor fleece	0.022
Cape 64's merino wool*	0.021
New Zealand 56's crossbred wool*	0.023
Blackface wool medulla	<0.02
Porcupine quill cortex	0.011
Porcupine quill medulla	0.002
Hen feather	0.018

* A non medullated keratin

The present results enable an upper limit to be assigned to the phosphorus of keratins present as phosphatide. The medulla constitutes only a small proportion of the total weight of wool. Barritt & King (1931) found 5.5% by microscopical observations, Jordan Lloyd & Marriott (1933) 13.5% in goat hair by isolation. If wool possesses the lipoprotein-cholesterol complex only on the surface of the medullary cells, its phosphorus content should be lower than that of the medulla. Such cells, isolated from blackface wool in amounts of 5-10 mg, after digestion with sulphuric acid, contained no phosphorus detectable by the Kuttner & Lichtenstein (1930) procedure. By this method, very small amounts (<0.02% on the weight of the medulla) should have been detected.

These results are in contrast to those of Stoves (1946 a, b), who obtained a 'positive tartaric acid-ammonium molybdate benzidine reaction' for phosphate on an extract prepared by refluxing medulla cells with 10% ethanolic sodium hydroxide for 5 hr. It is difficult to explain the difference between these two results, unless the lipoprotein-cholesterol complex is only found in a very few types of medullated fibres. Similar amounts of medulla cells of the rachis of hen feather also contained no detectable phosphorus, and the medulla of porcupine quill only a small amount. In contrast, the cortex of the quill had a definite phosphorus content. The reverse would be expected if the medulla contained appreciable amounts of lipoprotein. It is interesting that medullary cells isolated from the rachis of hen feather, or from porcupine quill by dissection, do not show the swelling and other reactions on treatment with tannic acid and sodium oleate observed by Stoves on medulla cells from Kolinsky fibres.

The cystine content of scoured keratins

Table 2 compares the analyses of the laboratory-scoured wool and kemp with those of the solvent-degreased specimens. The similarity of the analyses indicates that the laboratory method of scouring

causes little destruction of the combined cystine of wool and kemp. A similar conclusion can be drawn from Table 3, which gives the analyses of commercially scoured wool, the cystine sulphur and total sulphur contents of the wool after treatment in the various bowls of the scouring set are similar to those of the original wool. The cystine of wool thus seems to be stable to a commercial scouring process.

Table 2 *The sulphur analyses of laboratory-scoured and solvent degreased wool and kemp*

(Results as percentage of protein weight)

	Total S	Cystine S	Methionine S
64's Cape wool			
(a) Scoured	3.45	3.14	0.12
(b) Solvent degreased	3.48	3.09	0.12
Radnor kemp			
(a) Scoured	3.10	2.45	0.22
(b) Solvent degreased	3.24	2.45	0.22

Table 3 *The sulphur analysis of wool, before, during and after a commercial scour*

	Temp. of bowl (°)	pH of bowl	Total S (%)	Cystine S (%)
Original wool	—	—	3.48	2.97
Wool emerging from 1st bowl	52	9.85	3.53	3.02
2nd bowl	48	10.35	3.52	2.88
3rd bowl	48	10.70	3.46	2.82
4th bowl	40	9.40	3.48	2.95
5th bowl	38	8.95	3.50	2.92
Wool emerging from dryer	—	—	3.48	2.91

These results contrast with those of Bailey (1937). He found that while wool and kemp, obtained from the raw fleece which had been degreased with solvents, gave analyses in which 98–99% of the total sulphur was accounted for as cystine and methionine, only 90% of the total sulphur of scoured wool could be accounted for in this way, and only 36% in the case of scoured kemp. The present scoured kemp sample in particular does not show the strikingly large percentage of sulphur which is not present as cystine or methionine shown by Bailey's sample. Unfortunately, Bailey's analyses were carried out on different specimens of wool and kemp, the scoured and solvent degreased samples being derived from different sources and possibly being of different types. Unless this author's kemp had received exceptionally severe damage during processing, its unusual analysis can probably be attributed to some other cause than alkali damage during scouring.

Virgin wools and other keratins which have been degreased with solvents, and show no obvious signs of damage, often contain varying amounts of sulphur which is not present as cystine or methionine. Bailey similarly found that 6% of the sulphur of edestin and 7% of the sulphur of gliadin could not be accounted for in this way. This sulphur does not seem to be derived from lanthionine, which might be formed by the action of alkali on the combined cystine, since two dimensional partition chromatographic analysis (Consden *et al.* 1944) of such wools

often shows no detectable amount of lanthionine. Schoberl & Rambacher (1940) have suggested that the extent of damage by scouring might be determined by comparing the total sulphur with the cystine content. This suggestion should be treated with caution, since their criterion cannot be upheld in the present instance.

The amino-acid composition of medulla from keratins

In view of the evidence that the medulla of wool and porcupine quill has a sulphur content different from that of the cortex, two dimensional chromatograms were run on acid hydrolysates of medulla to obtain some idea of their amino acid composition. The chromatogram derived from the medulla of blackface wool differed from that of normal wool. For the medulla, the spots corresponding to glutamic and aspartic acids were strong, as were those corresponding to the basic amino acids. The leucine spot

was very strong, but those of the remaining amino-acids were weak. Similar chromatographic analysis was made on the medulla of porcupine quill. The spots corresponding to glutamic acid, leucine, valine, alanine and glycine were very strong, those of the remaining amino acids being very weak, many not being present in sufficient amount to give a readily observed spot. The chromatogram given by the cortex of the same quill (the quill tip) differed greatly from that of the medulla and closely resembled that given by normal wool. Analysis showed the medulla of a porcupine quill to contain 0.66% of disulphide + thiol S, as compared with a value of 1.47% for the cortex, which had a total sulphur content of 1.97% (Bekker & King (1931) found that the medulla of porcupine quills had a total sulphur content of 1.24% as compared with a value of 1.50% for the cortex).

DISCUSSION

Previous work had indicated that the medulla of keratins has a lower sulphur content than the cortex. The present results support this conclusion and provide evidence that the medulla differs in composition from the cortex with respect to other amino acids. The cystine of a heavily medullated kemp fibre, however, considered as a whole, does not

seem to differ from that of a normal keratin in its stability to the alkalis used in scouring. On the other hand, the treatment with sodium hydroxide used in isolating the medulla from wool probably involves some chemical degradation (Jordan Lloyd & Marriott, 1933, Stoves, 1946a). The very low sulphur content of the medulla of goat hair, found by Jordan Lloyd & Marriott (1933), may in part be due to the destruction by alkali of any cystine present. The medulla of porcupine quill can be isolated without such treatment.

The X ray diagram of medullary cells differs from that given by the cortex (Rudall, cited by Stoves, 1946a). Keratins in general give two types of X ray diagram, wool and hair an α keratin diagram, corresponding to folded polypeptide chains, while feather keratin gives a β keratin diagram, corresponding to almost fully extended polypeptide chains (Astbury & Marwick, 1932). The X ray diffraction pattern of medulla cells from porcupine quill shows the presence of β keratin and some unidentified material, the cortex gives a well defined α keratin diagram (MacArthur, 1943).

The main polypeptide chains of the medulla of porcupine quill are thus in the extended condition, in contrast to the folded chains of the cortex, and it is noticeable that the amino acids which are pre dominant in the former are those with non polar, often short, side chains, i.e. glycine, alanine, valine and leucine. This might suggest that one factor favouring the α or β keratin structure in nature may be the proportion of amino acids with relatively short side chains present. Astbury has pointed out that the structure of feather keratin may be thought of as intermediate between the structure of hair keratin (folded polypeptide chains) and that of silk fibroin (fully extended polypeptide chains). The latter protein contains a large proportion of short (glycine and alanine) side chains, and hence the polypeptide chains can pack closely together and assume an extended form. In α keratin, which has a large number of polar side chains, the polypeptide chain assumes a folded configuration. As a generalization, it is possible that β keratins contain a higher proportion of amino acids with short non polar side chains than α keratins, and that the larger pro

portion of longer side chains favours the α configuration. The chromatographic analysis of porcupine quill tip (α -keratin) and tortoise shell and snake skin, both of which give a β keratin X ray diagram, lent some support to this idea. Tortoise shell seems to be richer in glycine, alanine, valine, leucine, serine and tyrosine than porcupine quill tip, whereas snake skin was richer in glycine, alanine, serine and threonine.

The difference between the reaction of the medulla and cortex of wool fibres towards 4N-sodium hydroxide is probably largely dependent on structural differences. The cortex of porcupine quill is similarly attacked more rapidly than the medulla. When pieces of quill are immersed in 4N sodium hydroxide at room temperature, a considerable part of the cortex has dissolved after 24 hr., leaving the medulla largely intact. In 3 days, however, a considerable part of the medulla has also dissolved. Similar differences were observed between other keratins. Thus a 56's New Zealand wool was almost completely dissolved in a few hours, while duck feathers were largely undissolved. In general, the keratin structure with the α configuration is attacked most rapidly. It may be that the fully extended polypeptide chains of the medulla can pack closer together than those of the folded chains of the cortex, and are less accessible and less affected by reagents such as sodium hydroxide.

SUMMARY

1 The phosphorus content of a number of medullated and non medullated keratins, and of the isolated medulla of keratins, has been measured. None contains more than traces of phosphorus.

2 The combined cystine of a medullated keratin (kemp) or a normal wool is stable to the alkali employed in scouring. Two dimensional partition chromatography indicates that the medulla of wool and porcupine quill differs greatly from the cortex in amino acid composition.

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Studies on the Liver Catalase of Normal and Cancerous Rats

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Although the investigation of the systemic effects of malignant tumours is likely to constitute a fruitful approach to the cancer problem, the difficulty has always been the distinction between true cancer effects and those of a secondary nature, due to the concomitant destruction or impairment of vital organs and their functions, to necrosis, infection or suppuration, to haemorrhage and similar accessory factors. One of the least ambiguous examples of a specific systemic cancer effect is the diminution of liver catalase activity in a tumour-bearing organism.

This decrease was first described by Brahn (1916), who determined the catalase concentration in the livers of human beings who had died from various forms of cancer, and was recently extensively studied by Greenstein (1942) and his associates (Greenstein & Andervont, 1942, 1943, Greenstein, Andervont & Thompson, 1942). Their work, which was carried out on rats with transplanted subcutaneous tumours and on mice with transplanted, spontaneous or induced tumours of various kinds, established the following facts: (1) The decrease of liver catalase is progressive with the growth of the tumour. The activity may fall to one tenth or one twentieth of the normal value within 2 or 3 weeks after transplantation of a rapidly growing tumour. (2) The effect is reversible and the liver catalase activity returns to normal after excision or spontaneous regression of the tumour. Thus, in rats from which a transplanted hepatoma had been removed the normal liver catalase activity was restored almost completely after 24 hr and fully after 48 hr. On reimplantation of the tumour the liver catalase activity dropped again and was again brought back to normal by a second excision. (3) Kidney catalase activity is depressed much less than that of liver, only in mice with spontaneous mammary tumours is it lowered as much as, or more than, liver catalase. Erythrocyte catalase is apparently not affected at all. (4) The fall of catalase activity in the liver is probably due to a decrease of catalase concentration. No evidence for the presence of a catalase inhibitor could be found in tumour extracts or in the liver or serum of tumour bearing rats. (5) The effect is specific in a twofold sense although a few other liver enzymes are also less active as a result of a tumour elsewhere, e.g. D amino acid oxidase (Westphal, 1943) or arginase (Greenstein, Jenrette, Mider & White, 1941, Weil, 1935), the reduction is less striking than that of catalase. The majority of liver enzymes are unaffected. On the other hand, as far as is known at present, the catalase effect is caused only by cancer. In particular, it is not simply due to the presence of growing tissue, since pregnancy or growing implants of embryonic tissue fail to induce it.

It is obviously of great interest to obtain a better understanding of this phenomenon. 'Excluding any form of radiation from the tumour as too far fetched, the tumour may produce the effects noted either by giving off some toxic product to the circulation, or else by abstracting from the circulation some material essential to the normal maintenance of the liver catalase' (Greenstein, 1947).

The experiments to be described were designed to test the hypotheses quoted above. The elaboration of toxic products by growing tumours has often been postulated. Such an assumption is not unreasonable in the face of the extensive degradation processes accompanying an uncontrolled aggressive growth, which not only destroys surrounding normal tissues, but in many cases outstrips its own blood supply. One might therefore expect protein breakdown products, such as proteoses and polypeptides, in the circulation, and there is indeed some evidence for this (Winzler & Burk, 1944). It has been suggested that the output of protein split products by the tumour leads to a state of generalized intoxication and is the cause of the cachexia and marasmus of cancer patients (Reding, 1938). The effect of parental injection of foreign protein and of protein split products on the liver catalase of normal rats was therefore investigated.

If the invasiveness and unco-ordinated growth of a tumour results in augmented protein breakdown, the high rate of new growth will, on the other hand, greatly increase the demand for nitrogenous building materials. It is well known that a growing tumour will satisfy these demands without any regard for the welfare of the host, and the decrease in the concentration of certain liver enzymes may be due to lack of supplies required for their resynthesis or even to an accelerated 'melting down' process. The extent of the latter would depend upon the supply of nutrient material, and thus upon the diet.

In spite of the severe secondary anaemia often induced by cancer, the content of iron and copper in liver and spleen is usually above normal (Sandberg, Gross & Holly, 1942). It is improbable therefore that it is lack of these ions which is responsible for the decrease in either haemoglobin or catalase. It cannot be due to simple inanition either, since starvation of normal or tumour bearing rats (Greenstein, Jenrette & White, 1941 b, Miller, 1947) leads to a fall of liver catalase activity only in proportion with the decrease

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of liver proteins generally. The fall of liver catalase activity might, however, be caused by the exaggerated protein requirements of a rapidly growing tumour. Consequently, the progressive decline of liver catalase activity in two series of cancer bearing rats maintained on a high or a low-protein diet was studied. For the high protein diet a content of 45% casein was chosen, as this is sufficient to ensure maximal growth, whereas the low protein diet contained 8% casein, an amount adequate for the maintenance of body weight or even a moderate rate of growth in normal rats.

EXPERIMENTAL

Materials and methods

Animals Rats of both sexes from two inbred strains, a black hooded and a brown and white strain, were used at the age of 2-3 months, when they weighed c 100-150 g. Their routine diet consisted of 'rat cubes' (North Eastern Agricultural Co-operative Society, Aberdeen) with an approximate protein content of 20%, and of occasional supplements of green cabbage leaves. The low protein and high protein diets had the following composition:

	Low protein	High protein
Casein (%)	8	45
Maize starch (%)	72	35
Arachis oil (%)	15	15
Salts (Wesson, 1932) (%)	5	5

Water was added to the mixture to make a thick dough. The following vitamin supplements were dissolved in 100 ml water: ascorbic acid 6 mg, pyridoxin 6 mg, riboflavin 10 mg, calcium pantothenate 20 mg, inositol 200 mg, nicotinamide 200 mg, choline chloride 600 mg. The solution (5 ml/rat) was added daily to the water used for mixing with the food. Once weekly a few drops of cod liver oil were added to the ration. The average amount consumed daily/rat was about 15 g. Drinking water was supplied *ad libitum*. The rats were put on the diet 1 week before their inoculation with the Jensen rat sarcoma.

Serum injections Sheep serum was inactivated by heating to 56° for 0.5 hr. It was then passed through a bacteriological filter, sealed aseptically in ampoules and stored at 0°. Intraperitoneal injections of 2 ml. were given daily for 3-4 weeks, and were well tolerated. In animals killed only a few hours after injection the injected fluid was usually completely resorbed, and there was no sign of local reaction. Five rats surviving after 4 weeks therefore received an increased daily volume of 5 ml. for a further 15-30 days. The total amount injected thus varied from 45 to 210 ml. of serum per rat. Since the results showed no variation according to the total quantity or period of injections they were grouped together.

Peptone injections A 10% solution of a commercial sample of bacteriological peptone was adjusted to pH 7.25 (glass electrode), and subsequently sterilized and stored in the same way as serum. It gave a voluminous precipitate on addition of saturated $(\text{NH}_4)_2\text{SO}_4$ solution, and presumably contained proteoses. Two ml. were administered daily by intraperitoneal injection for 40-83 days, corresponding to a total of 80-166 ml. of peptone solution. Here, again, the results were grouped together, since no difference was

apparent between animals killed at the beginning and those killed at the end of the period. The peptone injections were not so well tolerated as the serum injections, resulting in loss of weight and listlessness. Reddening and hyperaemia of the peritoneal membranes were frequently observed at autopsy.

Transplantation of tumours The Jensen rat sarcoma (J.R.S.) was used for transplantation. The original tumour was obtained from the Imperial Cancer Research Fund Laboratories. Regressions were frequent in the beginning, but diminished after a few passages in our strains. Although a few experiments were performed on rats bearing slow growing or regressing tumours, these were not included in the results.

Preparation and standardization of liver extract After decapitation and exsanguination of the animal, the liver was removed and a weighed amount (c 7 g if available) was thoroughly homogenized with a little distilled water in a glass homogenizer (Potter & Elvehjem, 1936). After adding more water, in all three times the weight of liver used, the homogenate was left in the refrigerator overnight and was then centrifuged at 2500 r.p.m. for 5 min. The supernatant liquid was diluted to a volume proportionate to the weight of tissue taken, i.e. 100 ml. for 7 g. of liver.

It is advisable to adjust the extracts to a definite protein content. For this purpose Greenstein, Jenrette & White (1941a) estimated the total N of the extract. We found it more convenient to use the rapid colorimetric estimation of protein with Folin & Ciocalteu's reagent. All extracts were accordingly diluted to a certain value of 'protein tyrosine', obtained by subtracting the 'non protein tyrosine' from the 'total tyrosine'. The total tyrosine was estimated in 0.2 ml. of extract, after addition of 5N NaOH (2 ml.) and reagent (3 ml.), the solution was made up to 50 ml. For the estimation of non protein tyrosine, 5 ml. of a tungstic acid filtrate of the extract were used. The light absorption was determined photoelectrically with a tricolour red filter and calibrated with a tyrosine standard. As shown in Table 1 there is a constant ratio between the protein tyrosine and the protein N.

Table 1. Content of tyrosine and nitrogen in liver extracts of normal rats

Extract	Total tyrosine (mg/ml)	Total N (mg/ml)	N/tyrosine
1	1.40	2.910	2.08
2	1.15	2.520	2.19
3	1.60	3.740	2.34
4	1.60	3.612	2.26
5	1.13	2.316	2.08
Mean 2.19			
Extract	Protein tyrosine	Protein N	N/tyrosine
1	1.280	2.826	2.21
2	1.060	2.348	2.21
3	1.442	3.491	2.42
4	1.479	3.398	2.30
5	1.040	2.167	2.13
Mean 2.25			

Estimation of catalase activity The catalase activity was determined by measuring the rate of H_2O_2 decomposition colorimetrically with titanium sulphate (cf. Eisenberg,

1943) This reagent was prepared as follows Anhydrous titanium dioxide (2 g) was heated on a sand bath with 200 ml. of conc H_2SO_4 at $155-165^\circ$ until a clear solution was formed (usually 3-4 hr) After cooling, the solution was poured into distilled water, diluted to 1 l and filtered through Whatman no 5 filter paper One vol of the reagent was mixed with 9 vol of H_2O_2 solution containing 0.005-0.05 mg/ml, and the ensuing colour, which was stable for at least 24 hr, was measured photoelectrically with a blue filter A calibration curve was determined with a solution of H_2O_2 , standardized by KMnO_4 titration

If the concentration of phosphate buffer in the yellow solution of pertitanic acid is higher than 0.01 M, a turbidity may appear This can easily be avoided by the addition of 0.1 vol of 10N H_2SO_4 without in any way affecting the colour development In practice this was seldom necessary, since the H_2O_2 concentration was usually sufficiently high to require further dilution

The catalase concentration of a liver extract was tested at three different concentration levels at 0° The H_2O_2 concentration was 0.02N in the majority of experiments, the solution also contained 0.04M phosphate buffer, pH 6.8 At time $t=0$, 1 ml of enzyme solution of required strength was added to 50 or 100 ml of H_2O_2 phosphate buffer solution in an ice bath After 3, 6, 9 and 12 min, sometimes also after 1 min, samples of 5 or 10 ml were withdrawn and rapidly mixed with 0.1 vol of 30% (w/v) trichloroacetic acid A sample of the filtered solution was used for the colorimetric assay The initial value of H_2O_2 concentration was determined after adding 1 ml of enzyme solution to a sample containing 50 ml of H_2O_2 buffer solution + 5 ml of 30% trichloroacetic acid and filtering The monomolecular reaction constant was calculated by the formula

$$k = \frac{2.3}{t} \log \frac{[\text{H}_2\text{O}_2]_{\text{initial}}}{[\text{H}_2\text{O}_2]_{t \text{ min}}}$$

Haemoglobin This was estimated by the Haldane method.

RESULTS

Effects of high and low-protein diets on growth The growth rate of tumour-bearing rats on the high- or low-protein diet was determined by weighing them before transplantation, and again at death, after excision of the tumour which was weighed separately From the data in Table 2 the following facts emerge (1) The growth of the tumour is accompanied by a

fall of the body weight of the host even on a high protein diet, but this is significantly more severe in the rats on the low-protein diet (2) The rate of tumour growth is significantly greater on the high than on the low protein diet (3) The faster rate of tumour growth more than compensates for the loss of body weight in the high-protein series, whereas the animals on the low protein diet show not only a fall of body weight, but also of total weight (4) If

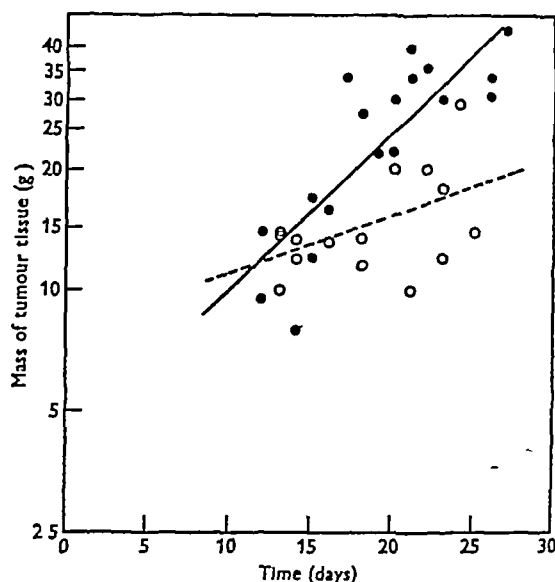


Fig 1 Regression (statistical) of tumour weight with time High protein diet dots and full line Low protein diet circles and broken line The straight lines are the linear regression lines

tumour weight is plotted against days, an exponential curve is obtained, rising steeply as the tumours become older If \log (tumour weight) is plotted against days the points are grouped about a straight line (Fig 1), the slope of which can be calculated by the method of least squares The slope of the high-protein curve is significantly steeper than that of the low protein curve, indicating a more rapid rate

Table 2 Effect of high- and low protein diets on body weight and tumour growth

(Figures represent mean values and their standard errors)

	Low protein diet	High protein diet	P*
No of observations	15	18	—
Days after transplantation	18.5	19.1	—
Final body weight (% of initial value)	77.0 \pm 2.64	90.5 \pm 2.60	<0.01
Tumour growth (g/days)	0.82 \pm 0.051	1.30 \pm 0.091	<0.01
Total weight at death (body + tumour, % of initial value)	91.5 \pm 2.28	109.1 \pm 1.71	<0.01
Regression coefficients			
Log (tumour weight) on days	0.0139 \pm 0.00688	0.0393 \pm 0.00676	0.015
Log (body weight)/(tumour weight) on days	-0.0315 \pm 0.00629	-0.0494 \pm 0.00473	0.03

* P = probability of difference being due to chance

Table 3 Protein tyrosine content of liver extracts

Exp	Days after transplantation (mean)	No of observations	Protein tyrosine (mean) (mg/ml)	Standard error of mean
Normal rats				
Cube diet	—	17	0.922	0.0178
High protein diet	—	6	0.975	0.0375
Low protein diet	—	8	0.833	0.0202
Injected with sheep serum	—	10	0.896	0.0200
Injected with peptone	—	5	0.923	0.0285
Tumour bearing rats				
Cube diet	22.70	19	0.710	0.0227
High protein diet	19.26	20	0.697	0.0160
Low protein diet	18.14	14	0.655	0.0182

of growth (5) When the logarithm of the ratio (body weight)/(tumour weight) is plotted against days and the linear regression curve calculated

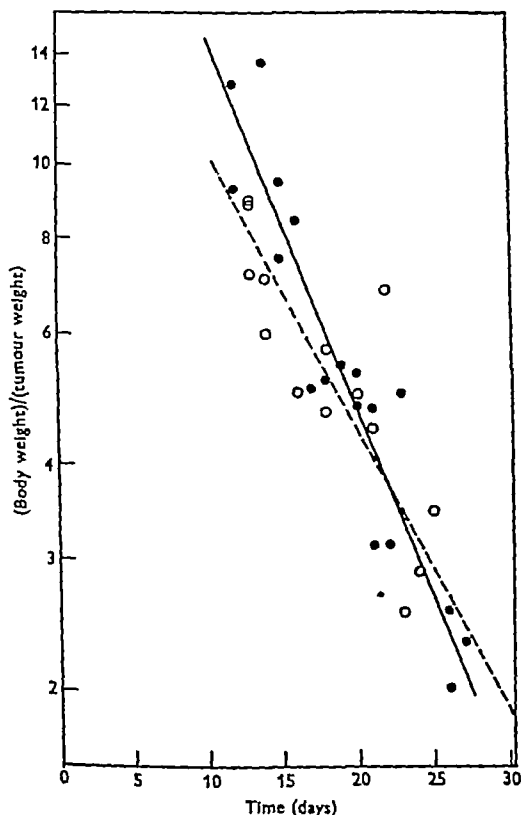


Fig 2 Regression of ratio (body weight)/(tumour weight) on days after transplantation. High protein diet dots and full line. Low protein diet circles and broken line. The straight lines are the linear regression lines.

(Fig 2), it is found that the difference between the high and low protein series, though still probably significant, is less than that between the curves representing tumour growth rates. This indicates

that the faster tumour growth on the high-protein diet is to some extent balanced by a smaller loss of body weight.

Changes in protein tyrosine of liver (a) *Normal rats* The concentration of protein tyrosine in liver extracts of normal rats on the standard diet showed very little individual variation. It was not significantly raised either by a high protein diet or by injections of serum or peptone (Table 3). On the other hand, there was a slight decrease in the rats on the low protein diet, the difference from the standard series being statistically significant ($P < 0.01$). The animals on the high- or low protein diets which were used for these analyses had previously been inoculated with J R S, but the tumours had failed to grow or had regressed. The analyses were performed 4–5 weeks after the start of the diets and not less than 1–2 weeks after the complete disappearance of tumours.

(b) *Tumour rats* The presence of a growing tumour invariably caused a considerable decrease of the protein-tyrosine concentration of liver extracts, which was not significantly affected by the protein level of the diet.

Table 4 Haemoglobin concentration

	No of observations	Mean Hb (%)
Normal rats		
Cube diet	8	79.6
High protein diet	6	85
Low protein diet	8	76.6
Tumour rats		
Cube diet	11	49.7
High protein diet	16	66.1
Low protein diet	14	54.6

Haemoglobin Estimations of haemoglobin were carried out in a number of cases and the results are shown in Table 4. There is a marked drop in tumour-bearing rats, as is well known (cf Taylor & Pollack, 1942). Whether the differences between the high and low protein series are significant seems doubtful in view of the high margin of error of the method.

There were, however, indications of a correlation between the degree of secondary anaemia and the size and age of the tumour

The catalase activity of liver extracts of normal and tumour bearing rats Catalase was measured at three concentration levels after 3, 6, 9 and 12 min. During this time the activity gradually diminished by 10–20%. In most experiments the enzyme concentrations were varied in the proportion of 1:2:4, the actual values depending on the presence of a tumour and its size. A value for the monomolecular reaction constant of $c = 0.1$ with the highest and of $c = 0.025$ with the lowest enzyme concentration was sought. With extracts of normal rats, final concentrations of 0.4–0.1 $\mu\text{g/ml}$ protein tyrosine were required, 1 ml of extract containing 20–25 $\mu\text{g/ml}$ protein tyrosine was added to 50 ml of substrate solution. With tumour rats, these concentrations had to be increased 5–10 fold. The H_2O_2 concentration in these experiments was 0.02N throughout.

There was a very satisfactory proportionality of k (the reaction constant) with enzyme concentration (Table 5), unaffected by the relative substrate concentration. In a number of experiments the enzyme concentration was varied in the proportion of 1:10:100, to test for a possible inhibitory effect of serum or peptone injections which might have manifested itself only at high enzyme concentrations. But the fairly close proportionality of k with enzyme concentration, even over this extreme range, makes the existence of such an effect very unlikely. The ratio of substrate/enzyme concentration was kept constant here in order to avoid an unequal degree of enzyme inhibition by excess substrate.

The satisfactory proportionality between enzyme activity and concentration, at any rate over a limited range, makes it possible to express enzyme

units in terms of activity and protein content. Catalase concentration has therefore been expressed in arbitrary units defined by the ratio k/mg protein tyrosine/ml. The mean value of the results obtained with the three different enzyme concentrations was taken as the unit content of a given extract.

Catalase concentration in the liver of non tumour rats The concentration of catalase in the liver of normal rats is fairly constant if extracts of equal protein concentration are compared. It is not significantly changed either by high or low protein diets or by injections of peptone solutions (Table 6). However, injection of sheep serum leads to a significant increase of catalase concentration. Not only the difference between serum and standard series, but also that between serum and peptone series, is significant ($P < 0.01$ and $P = 0.01$ respectively). This is the more remarkable as the protein concentration of liver extracts was not raised by serum injections. On the other hand, although rats on the low-protein diet show a decrease of liver protein, the ratio between protein and catalase concentrations remains constant.

Since there was no essential difference between them, the first four experiments of Table 6 were grouped together to provide a standard of comparison with the catalase concentration in the liver of tumour rats.

Catalase concentration in the liver of tumour bearing rats Where our experiments covered the same ground as those of Greenstein and his associates the results were in full agreement with theirs. A fall in catalase activity was found, which was progressive with the growth of the tumour (in extreme cases, to

Table 5 Proportionality of the monomolecular reaction constant k with enzyme concentration

Series	No of observations	Protein tyrosine (mg/ml liver extract)	H_2O_2 concentration (N)	Mean value of k after time (min)		
				3	5	12
Normal rats, cube diet	17	0.02	0.02	0.1043	—	0.0906
		0.01	0.02	0.0520	—	0.0460
		0.005	0.02	0.0276	—	0.0232
Normal rats, injected with serum or peptone	8	0.25	0.2	1.20	1.10	—
		0.025	0.02	0.178	—	0.147
		0.0025	0.002	0.0202	—	0.0158

Table 6 Concentration of liver catalase in normal rats

(Values in arbitrary units/ml, see text)

Exp	No of observations	Catalase concentrations (mean values) \pm standard errors of mean after time (min)	
		3	12
Cube diet	17	5.21 ± 0.316	4.57 ± 0.267
High protein diet	5	5.55 ± 0.438	4.34 ± 0.404
Low protein diet	8	5.20 ± 0.190	4.28 ± 0.163
Injected with peptone	5	6.01 ± 0.353	4.93 ± 0.280
Injected with serum	10	7.58 ± 0.427	6.24 ± 0.282
First 4 experiments combined	35	5.37 ± 0.301	4.52 ± 0.265

only 5% of normal) There were, however, large individual variations, even when similar stages of tumour growth were compared

If a correlation is to be sought between the size of the tumour and the concentration of liver catalase, the absolute size of the tumour might be regarded as of less importance than the tumour size relative to the size of the host, as expressed by the ratio (body weight)/(tumour weight) If catalase concentrations are plotted against this ratio, an exponential curve is again obtained, and the points are grouped along a straight line when plotted on a semi logarithmic scale (Fig 3) In spite of the wide scatter, the regressions of catalase concentration on the ratio of (body

weight)/(tumour weight) are significantly different from zero in the high- and low protein experiments In the experiment with rats on the standard diet the (statistical) regression is hardly significant ($P=0.09$) This is probably because the points were not sufficiently spread out along the x axis

It is quite obvious from an inspection of Fig 3, and is confirmed by statistical analysis of the regression coefficients (Table 7), that the effect of tumour growth on the concentration of liver catalase is not significantly influenced by the level of protein

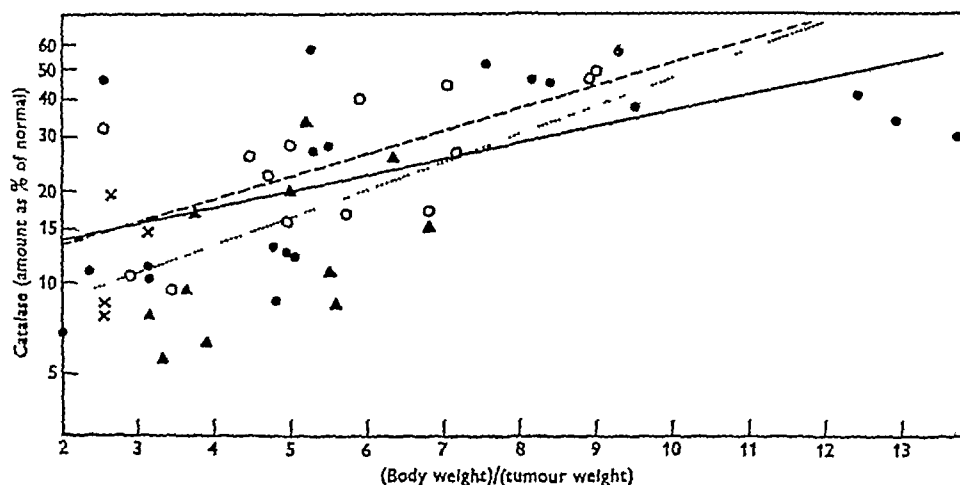


Fig 3 Regression of liver catalase concentration (in % of normal concentration) on ratio (body weight)/(tumour weight) Standard diet triangles and dotted line Standard diet and serum injections crosses. High protein diet dots and full line Low protein diet circles and broken line The straight lines are the linear regression lines

Table 7 Concentration of liver catalase in tumour-bearing rats

Series	No of observations	(Body weight)/(tumour weight) (mean)	Days after transplantation (mean)	Catalase units (% normal) (mean)	Regression coefficient of catalase concentration on (body weight)/(tumour weight)
Cube diet	15	4.20	22.14	13.95	0.0747 ± 0.0408
High protein diet	20	6.53	19.05	29.22	0.0517 ± 0.0165
Low protein diet	14	5.61	18.14	27.65	0.0734 ± 0.0254

weight)/(tumour weight) are significantly different from zero in the high- and low protein experiments In the experiment with rats on the standard diet the (statistical) regression is hardly significant ($P=0.09$) This is probably because the points were not sufficiently spread out along the x axis

It is quite obvious from an inspection of Fig 3, and is confirmed by statistical analysis of the regression coefficients (Table 7), that the effect of tumour growth on the concentration of liver catalase is not significantly influenced by the level of protein

In view of the definite increase of liver catalase observed in normal rats after intraperitoneal injections of sheep serum, the effect of serum injections on tumour bearing rats was of interest Four rats (marked \times in Fig 3) were given daily intraperitoneal injections of 5 ml of sheep serum, starting 11 days after transplantation when the tumours were easily palpable The injections were continued for 8-10 days, so that a total amount of 40-50 ml was injected There was no obvious effect on either the rate of tumour growth or the concentration of liver

catalase Two more series each consisting of five rats received serum injections from the fourth and fifth day after transplantation onwards In all ten animals regression of the tumours occurred In two

Table 8 *Concentration of liver catalase in tumour-bearing rats*

Days after trans- plantation	(Body weight)/ (tumour weight)	Catalase concentration (% normal)
Fast growing, early tumours		
13	8.9	46.9
13	9.0	49.7
12	9.3	57.3
12	12.9	33.8
14	7.06	44.4
Slow growing or regressing tumours		
21	29.0	24.3
23	11.0	27.6
23	12.2	15.9
26	7.15	10.3
27	14.3	35.8

control series the tumours grew in 3 out of 5 and in 3 out of 3 animals It would be premature to draw any conclusions from the small number of animals in these experiments, especially in view of a tendency towards regression in the controls, but the results suggest an inhibitory effect of early serum injections on the growth of transplanted J.R.S. This point is being further investigated

DISCUSSION

The fact that the presence of a growing tumour leads to a progressive fall of the concentration of liver catalase far in excess of the lowering of liver proteins generally need not necessarily be inconsistent with an unspecific inhibition of protein synthesis such as would result from the appropriation of available amino acids by the tumour If the rate of disintegration and regeneration were much faster for catalase than for other liver enzymes, the specific effect on catalase could be interpreted as the outcome of an unspecific inhibition of protein synthesis There are, indeed, indications that liver catalase has a short life (1) it has a high rate of wastage during enzymic activity, (2) a high rate of resynthesis after removal of a transplanted tumour, (3) in contrast to erythrocyte catalase, it contains a verdohaemochromogen group which, as Lemberg & Legge (1943) suggest, is due to an *in vivo* oxidation

The experiments on the effect of high- and low-protein diets have provided no support for this mechanism If a shortage of amino acids were the cause, one would have expected at least a delay in the decline of catalase concentration as a result of the high protein feeding That the fall of body weight is considerably less severe on the high- than on the

low protein diet shows that the higher protein level is not entirely ineffectual, and the lack of response of the catalase concentration suggests that other causes than protein shortage are operating This conclusion, however, is not yet final An effect might appear if the rate of protein synthesis could be speeded up, e.g. by parenteral administration of essential amino acids in high dosage Alternatively, an excess of one or more particular amino acids may be required The observation that serum injections raised the level of catalase concentration in normal livers, whereas peptone injections were ineffective, may point in this direction Peptone is particularly deficient in tryptophan, and it would be interesting therefore to study the effect of massive doses of this amino acid

The possibility that the lowering of catalase concentration in the liver is due to a toxic principle elaborated by the tumour and given off into the circulation also deserves further study It is true that Greenstein (1943) found no indication for the presence of a catalase inhibitor in tumour tissue or in other tissues of cancerous animals But the possible existence of an inhibitor of catalase synthesis or a catalyst of catalase destruction has not yet been exhaustively explored Our experiments show that prolonged parenteral injection of foreign protein or of protein breakdown products does not duplicate the tumour effect in normal rats A prolonged treatment of normal rats with tumour extracts might now be attempted Greenstein (1943) carried out some experiments on these lines, but he gave only one injection of 1 or 2 ml. of extract, whereas it may be necessary to extend the injections over a period of several weeks Moreover, his extracts, which were prepared with saline, were probably devoid of nucleoproteins, and it is feasible that the toxic factor is associated with this fraction

SUMMARY

1 The concentration of liver catalase in normal rats and in rats bearing a transplanted Jensen rat sarcoma was determined The rats were maintained on one of three diets (a) a standard diet of rat cubes (20% protein), (b) a high protein diet (45% casein), (c) a low-protein diet (8% casein) Additional experiments were performed on rats which received daily intraperitoneal injections of sheep serum or of a 10% solution of peptone for several weeks

2 The high-protein diet caused a significantly faster growth of the tumours and a smaller loss of body weight in tumour bearing rats than the low protein diet The protein content of the liver was slightly reduced in normal rats on the low protein diet, it was considerably lowered in all tumour bearing rats, irrespective of diet

3 The catalase concentration in normal rat liver extracts of equal protein content was not significantly changed, either by different protein levels in the diet or by peptone injections. It was raised, however, by serum injections.

4 In confirmation of the work of Greenstein and his group (1941-3) it was found that the growth of a transplanted tumour leads to a fall of liver catalase concentration which, in extreme cases, may reach 5% of normal. There was a very marked individual scatter even in similar stages of tumour growth.

5 The catalase concentration when plotted

against (body weight)/(tumour weight) was found to follow an exponential curve.

6 The protein level of the diet had no significant effect on the course of the process, even in the initial stages. Serum injections had no effect on tumour-bearing rats when started 11 days after transplantation. When started 4 or 5 days after transplantation they were followed by regression of the tumour.

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The β -Glucuronidase Activity of Ox Spleen and the Assay of β -Glucuronidase Preparations

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The existence in animal tissues of an enzyme which hydrolyzes β glucuronides was first reported by Sera (1915). In 1934, Masamune described a method of preparation of active extracts of this enzyme from ox kidney having pH optima for the hydrolysis of methylglucuronide and phenylglucuronide in citrate buffers at 5.3 and 5.3-5.6 respectively.

Oshima (1934) reported data for the glucuronidase content of various tissues of the dog and the ox, rich sources being liver, spleen, kidney, ovary, testes and thymus. In 1936 Oshima introduced improvements into the method of Masamune (1934), which were claimed to give a purer preparation. For this preparation the pH optimum for the hydrolysis of methylglucuronide in acetate buffer was quoted as 5.0-5.2. In the work reported up to that time, little

or no data were presented concerning the degree of purification or the losses at each stage of the preparation of β glucuronidase.

The first quantitative study of the purification of ox spleen glucuronidase was reported by Fishman (1939a). By employing ammonium sulphate fractionation after preliminary treatment, Fishman achieved an eightfold concentration of the enzyme and a 140 fold purification. There was, however, an overall loss of 84% by this method. Fishman (1939b) reported that the pH optima of his preparation in an acetate buffer were 5.0 for *l* methylglucuronide, 4.4 for borneolglucuronide and 4.3 for oestriolglucuronide.

Florkin, Crismer, Duchateau & Houet (1942) also studied glucuronidase, and demonstrated the reversibility of the hydrolysis of borneolglucuronide *in vitro*.

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A much improved method of preparation of α -spleen glucuronidase was reported by Graham (1946) in which a 400 fold concentration and a 315 fold purification of the enzyme was achieved. Graham found that a preliminary autolysis of an aqueous extract of acetone extracted tissue caused an increase in the amount of the enzyme, and that this was accompanied by a liberation of reducing material. These facts were shown to be complicating factors in the assay of crude preparations of the enzyme. It was also demonstrated that pH control was an important factor during the separation of the enzyme from inactive protein by ammonium sulphate fractionation. Graham, however, reported no data on the pH optima or reaction kinetics of his preparation.

Talalay, Fishman & Huggins (1946) reported data concerning the hydrolysis of phenolphthalein glucuronide by fairly crude preparations of glucuronidase from pooled livers, kidneys and spleens of mice. The pH optimum in acetate buffer for this substrate was shown to be 4.5. Data on the kinetics of the reaction were also presented. Talalay *et al* (1946) and Fishman & Talalay (1947) have used phenolphthalein glucuronide and pH 4.5 in a method of assay of the glucuronidase content of various tissues of the rat.

In the present work further data on the preparation and purification of α -spleen glucuronidase are presented. The data reveal that two enzymes which hydrolyze *l*-menthylglucuronide are present in α -spleen, and that these two enzymes, which can be separated, have different pH optima. A modified technique for the assay of glucuronidase preparations using *l*-menthylglucuronide is presented along with assay methods employing phenylglucuronide and phenolphthalein glucuronide.

A preliminary report of this work has already appeared (Mills, 1947).

EXPERIMENTAL

Reagents

l-Menthylglucuronide, prepared by the biosynthetic method of Quirk (1924).

Phenylglucuronide, prepared by the method of Masamune (1933) as modified by Williams (1945), m.p. 160° (uncorr.).

Phenolphthalein glucuronide, prepared by the method of Talalay *et al* (1946).

D Glucuronic acid, prepared by the method of Williams (1940), m.p. 176–177° (uncorr.), $[\alpha]_D^{20} +20^\circ$.

Trichloroacetic acid (British Drug Houses Ltd.), 20% (w/v) aqueous solution.

Alkaline ferricyanide solution, 5 g $K_3Fe(CN)_6$ (A.R.) and 10 g anhydrous Na_2CO_3 in water to 1 l. This is stable for 1 month in a dark bottle, after that time the blank value in the reducing test increases considerably.

Sodium carbonate, 10% (w/v) aqueous solution.

Ceric sulphate, approx 0.1N solution, prepared according to Miller & Van Slyke (1930), and diluted 1 in 10 in H_2SO_4 for daily use.

Sulphuric acid, 18N (A.R.).

Lissamine green (British Drug Houses Ltd.), 0.05% (w/v) aqueous solution.

Phenol reagent, prepared as described by Folin & Ciocalteu (1927).

All pH measurements were carried out with the glass electrode and Cambridge pH meter.

ASSAY OF GLUCURONIDASE PREPARATIONS

(a) Using *l*-menthylglucuronide

In the methods of Fishman (1939a) and Mills (1946) the neutralization of excess trichloroacetic acid, after the removal of precipitated protein from the enzyme digests, was accomplished by *N*-NaOH in the presence of phenolphthalein. This method gave rise to high blank values in the subsequent ferricyanide reduction. The method of Levvy (1946), which employs an alkaline copper tungstate deproteinization, was found to be unsatisfactory if the pH of the enzyme digest was less than 4.6. This criticism applies also to a cadmium hydroxide precipitation, a preliminary exact neutralization of the digest being necessary in both cases to give reliable results.

The most satisfactory procedure so far found involves a trichloroacetic acid deproteinization followed by addition of excess sodium carbonate. It was found that wide variations in the sodium carbonate concentration were allowable in the ferricyanide reduction (cf. Levvy, 1946).

Method. Acetate buffer (0.2M, 1.0 ml), 0.5 ml of 0.02M *l*-menthylglucuronide solution (pH c. 5) and 0.5 ml of a suitable dilution of the enzyme solution are incubated in 15 ml centrifuge tubes for 2 hr at 38°. The reaction is then stopped and protein removed by the addition of 1 ml of 20% trichloroacetic acid followed by centrifugation, 2 ml of the supernatant fluid are transferred to 4 × 4 in Pyrex tubes and 1 ml of 10% Na_2CO_3 solution followed by 1 ml of alkaline ferricyanide solution added. The tubes are closed with glass bulbs, heated in a boiling water bath for 15 min and then cooled in running water. H_2SO_4 (18N, 0.25 ml) is added, followed by 1 drop of 0.05% Lissamine green solution, and the mixture titrated with c. 0.01N ceric sulphate solution using a 2 ml micro burette. Stirring is performed by a slow stream of N_2 , and a daylight lamp is used for the titration.

The ceric sulphate is standardized against D-glucuronic acid and results expressed as glucuronic acid by employing the appropriate correction factor. Controls are carried out using buffer and enzyme solution alone, since it was found that hydrolysis of the substrate in the absence of glucuronidase at pH 3–7 could be neglected.

By using unboiled enzyme extracts in the control tubes interference caused by the increase in reducing material on incubation of crude extracts is avoided. Graham (1946) first demonstrated this increase in reducing material and some typical data are presented in Fig. 1. This method does not avoid, however, the increase in enzymic activity which accompanies the increase in reducing material on incubation of such crude extracts. Neither increase occurs in extracts after precipitation with ammonium sulphate.

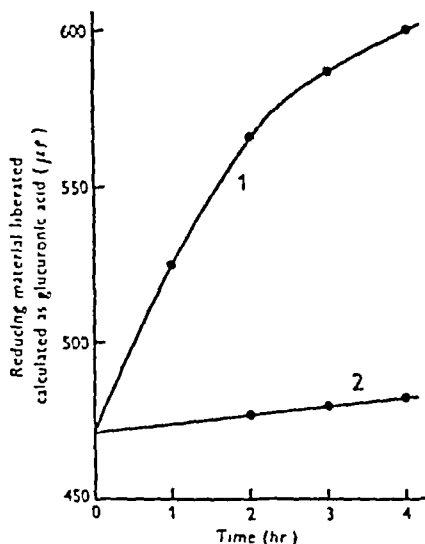


Fig. 1 Liberation of reducing material (calculated as glucuronic acid) during the incubation of 2 ml samples of a crude spleen extract at 38°. 1, unboiled extract, 2, boiled extract.

It was also found that the presence of ammonium sulphate at a concentration of 0.05*N* or less in the enzyme digests did not affect the results of assay.

The glucuronidase reaction was found to follow the equation

$$k = 1/t \log_e a/(a-x)$$

at low substrate concentrations, where t = time in hours, a = original amount of substrate and x = amount of substrate split in time t .

Unit of glucuronidase activity. The unit of glucuronidase activity (menthyl unit) was taken as the activity associated with a value of $k = 0.1$ in the above equation, under the conditions specified.

(b) Using phenylglucuronide

The method is based upon the determination of phosphatase using phenylphosphate (King & Armstrong, 1934; Folley & Kay, 1935). Phenylglucuronide is incubated with the enzyme preparation in a buffer solution, and after a standard time the reaction stopped and the phenol estimated using the reagent of Folin & Ciocalteu (1927).

Method. Acetate buffer (0.2*N* 1.0 ml), 0.5 ml of 0.02*N* phenylglucuronide (Na salt) and 0.5 ml of enzyme solution are incubated in 15 ml centrifuge tubes for 1 hr at 38°. 2 ml of a 1 in 3 dilution of the Folin Ciocalteu reagent are added and the protein precipitate removed by centrifugation. 3 ml of the supernatant fluid are added to 3 ml of 10% Na_2CO_3 and the mixture incubated at 38° for 40 min. Water (2 ml) is added and the blue colour estimated in the Spekker photoelectric absorptiometer using Ilford no. 608 red filters. The phenol liberated is estimated by using a calibration curve determined with phenol solutions treated as in an enzyme assay.

Controls were set up in which the buffer and substrate were incubated together for 1 hr at 38° and then the enzyme, immediately followed by the diluted Folin reagent added at the end of this time.

Since the liberation of phenol was found to follow a linear course with time and to be directly proportional to the enzyme concentration, the amount of phenol liberated is, therefore, directly related to the amount of enzyme present.

1 phenyl unit = 1 μg phenol liberated/hr under the above conditions.

(c) Using phenolphthalein glucuronide

The method is essentially that of Talalay *et al* (1946) with the difference that the enzyme digests consist of 0.5 ml of 0.2*N* acetate buffer, 0.2 ml of 0.005*N* phenolphthalein glucuronide (Na salt) and 0.3 ml of enzyme solution, and that protein is removed with trichloroacetic acid before bringing the pH to 10.4. The colour is estimated in the Spekker photoelectric absorptiometer using an Ilford 605 yellow green filter.

1 phenolphthalein unit = 1 μg phenolphthalein liberated/hr under the above conditions.

Determination of protein nitrogen

The protein in 1 ml samples was precipitated with 1 ml of 20% trichloroacetic acid and the mixture heated to 80° for 10 min. The precipitate was removed by centrifugation and washed on the centrifuge with 5 ml of 5% (w/v) trichloroacetic acid. If ammonium sulphate was present in the original sample, the washing was repeated a further four times. The protein precipitate was dissolved in 3 ml of 18*N* sulphuric acid and the protein N estimated, after digestion, by the micro Kjeldahl method of Ma & Zunzaga (1942), employing the Markham (1942) distillation unit.

PREPARATION OF β GLUCURONIDASE

The finding of Graham (1946) that acetone precipitation of the enzyme from aqueous solution followed by extraction of the precipitate with water, as employed by Fishman (1939*a*), gave rise to serious

losses was confirmed in the present work. It was also found, in accordance with Graham (1946), that by extracting the tissue pulp with two successive lots of 2 vol of acetone and subsequent extraction of the solid residue with water, one could avoid these serious losses. Ethanol precipitation, as employed

influenced by both these factors. It is quite obvious therefore that the results of ammonium sulphate fractionation of protein mixtures will be significantly influenced by variations in pH and temperature, factors which are often neglected during salt fractionation of proteins.

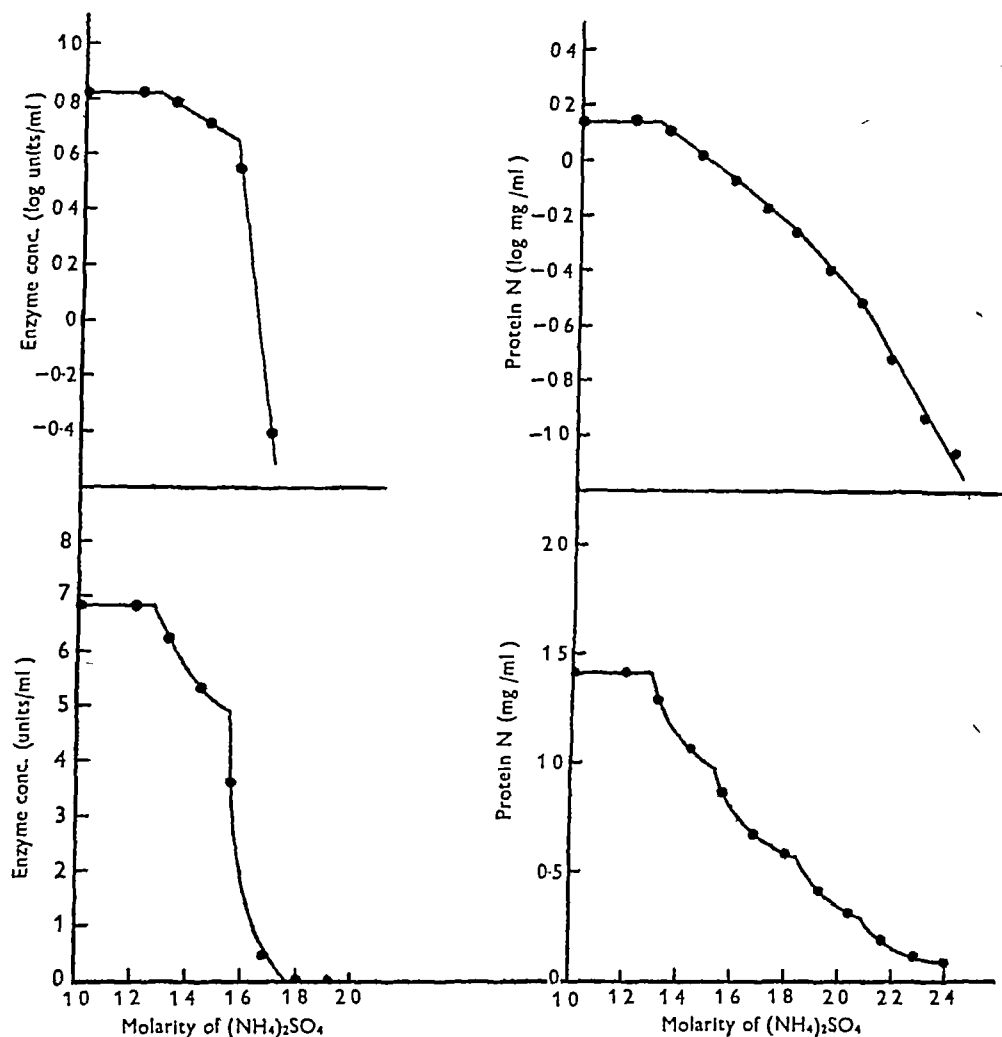


Fig 2 Variable solvent solubility test on spleen extract at stage C of preparation, carried out at pH 5. Ammonium sulphate used as precipitant and glucuronidase assayed at pH 5 using *l* menthylglucuronide

by Masamune (1934) and Oshima (1936), also gave rise to as much as a 50 % loss of activity.

In the method of Fishman (1939a) there was no control of the pH during ammonium sulphate fractionation. Cohn (1925) has shown that the solubility of proteins in salt solutions obeys the equation

$$\log S = \beta - kI,$$

where S = solubility, I = ionic strength, and β and k are constants. Cohn found that while k is independent of temperature and pH, β is markedly

Graham (1946) took pH into account during ammonium sulphate fractionation, but did not indicate how the limits employed were derived.

In order to obtain precise data in the present work the variable solvent solubility test as defined by Falconer & Taylor (1946) was applied to crude glucuronidase solutions at two different pH values.

Samples (4 ml) of a glucuronidase solution, adjusted to the requisite pH, were introduced into a series of 15 ml centrifuge tubes and saturated (NH₄)₂SO₄ at the same pH added to give the required ionic strength. The tubes were

allowed to stand at 0° for 18 hr and the precipitates removed by centrifugation at 0°, were dissolved in water and assayed at pH 5 for glucuronidase activity using 1 month old glucuronide. Protein N determinations were carried out on the supernatant solutions.

The data obtained at pH 5.0 and 7.0 (Figs 2 and 3 respectively) reveal that two proteins exist in spleen

ditions of temperature and pH which make $\beta_A - \beta_B$ a maximum are optimum for separation.

These constants must be determined on the pure phases to give absolute values, but the direction of difference can be obtained from the solubility test. If in the present work the fraction precipitated at lower salt concentration gives l_A and the other

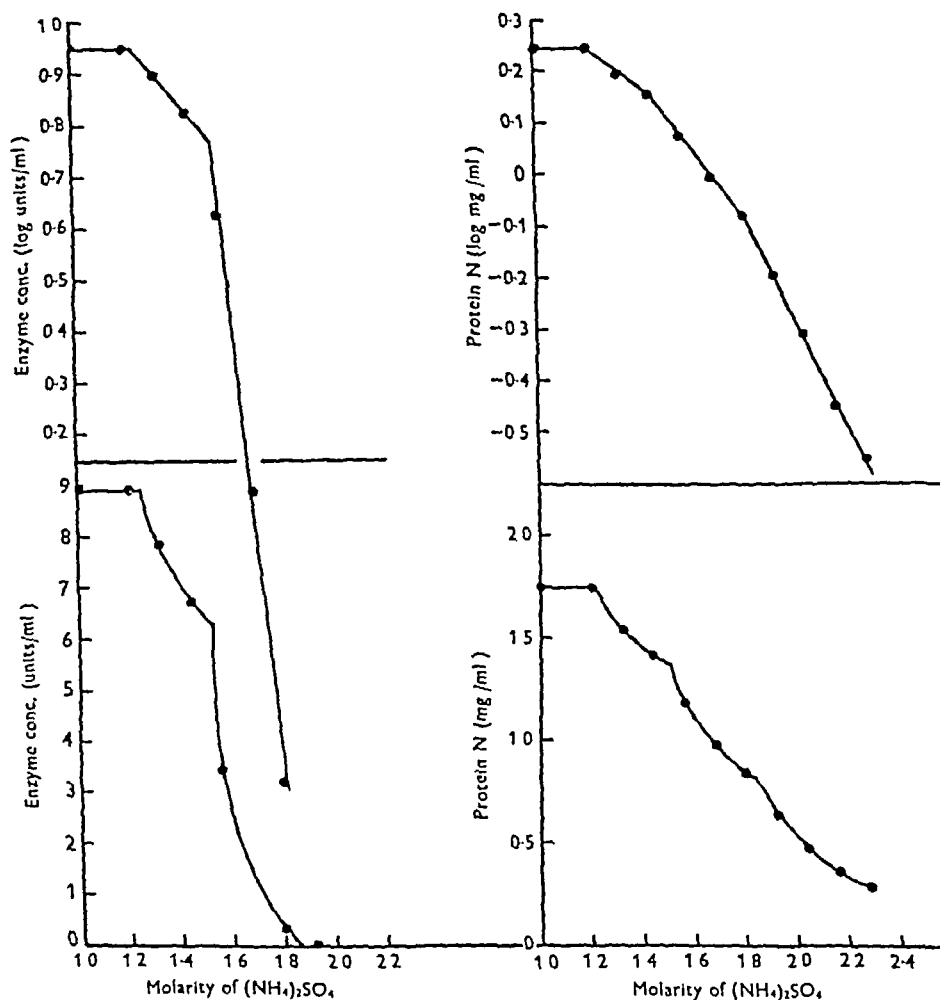


Fig 3 Variable solvent solubility test on spleen extract at stage C of preparation, carried out at pH 7. Details as for Fig 2

extracts, both having glucuronidase activity. Falconer & Taylor (1947) indicated methods of separation of two phases (A and B) based upon determination of the constants β and l . They stated that if for two fractions l_A is greater than l_B then a dilution of the original solution aids separation of phase A in a pure form. If l_B is greater than l_A then concentration aids in obtaining A in pure form. Also con

fraction l_B then it is obvious from the data that l_B is greater than l_A .

A method of purification of glucuronidase was worked out using the data obtained above. Throughout the procedure which follows, the pH was controlled at each stage by means of a glass electrode, and all operations requiring temperature control were carried out in a cold room at 0°.

Stage A Fresh α spleens were stripped of fat and finely minced. Redistilled acetone (3 l) was stirred into 1.5 kg of this mince and the mixture filtered and sucked dry. The pressed filter cake was stirred with a further 3 l of acetone and the mixture filtered with suction and pressed well. The filter cake was broken up and dried in air at room temperature for about 30 min. The spleen powder was stirred with 3 l of water for 1 hr, strained through gauze, clarified in the Sharples super centrifuge and dialyzed against running tap water for 24 hr (solution A).

Stage B Solution A was adjusted to pH 5.0 with 2N acetic acid and M acetate buffer (pH 5.0) added (20 ml/l). A voluminous precipitate formed. The mixture was allowed to stand at 38° for 4 hr and the precipitate removed in the Sharples super centrifuge. The filtrate was finally clarified by filtration using Standard Super Cel (Johns Manville Co Ltd) (solution B).

Stage C Solid $(\text{NH}_4)_2\text{SO}_4$ was stirred into solution B to 60% saturation and the mixture allowed to stand at 0° for 18 hr. The supernatant liquid was siphoned off and the precipitate removed by filtration using Standard Super Cel (10 g/l). The filter cake was sucked dry and then extracted five times with 100 ml portions of water. The extracts were combined and dialyzed against running tap water for 24 hr (solution C).

Stage D Solid $(\text{NH}_4)_2\text{SO}_4$ was added to solution C to 1.2M and the pH adjusted to 5.0 with 2N acetic acid. The mixture was allowed to stand at 0° for 6 hr, the precipitate removed at the centrifuge and discarded. To the solution $(\text{NH}_4)_2\text{SO}_4$ was added to 1.8M, the pH readjusted to 5.0 and the mixture allowed to stand at 0° for 18 hr. The precipitate was removed at the centrifuge at 0°, dissolved in water and dialyzed against running tap water for 24 hr (solution D). This stage removes a considerable amount of inactive protein.

Stage E Solution D (about 500 ml) was adjusted to pH 5.0 with N acetic acid, and saturated $(\text{NH}_4)_2\text{SO}_4$, previously adjusted to pH 5.0, was added to 1.26M. The mixture was allowed to stand at 0° for 6 hr and the precipitate removed at the centrifuge at 0° and discarded. To the supernatant liquid saturated $(\text{NH}_4)_2\text{SO}_4$ at pH 5 was added to 1.54M, the solution allowed to stand at 0° for 18 hr, the precipitate removed at the centrifuge at 0°, dissolved in 25 ml of water and dialyzed against running water for 24 hr (fraction I). To the supernatant liquid from the above precipitation, $(\text{NH}_4)_2\text{SO}_4$ at pH 5.0 was added to 1.78M, the

mixture allowed to stand at 0° for 18 hr, the precipitate removed at the centrifuge at 0°, dissolved in 200 ml water, and dialyzed against running water for 24 hr (fraction II).

Stage F Fraction I was reprecipitated with saturated $(\text{NH}_4)_2\text{SO}_4$ at pH 5 between 1.26 and 1.52M at 0°, and the precipitate was washed on a centrifuge at 0° with 1.52M $(\text{NH}_4)_2\text{SO}_4$ at pH 5, dissolved in the minimum amount of

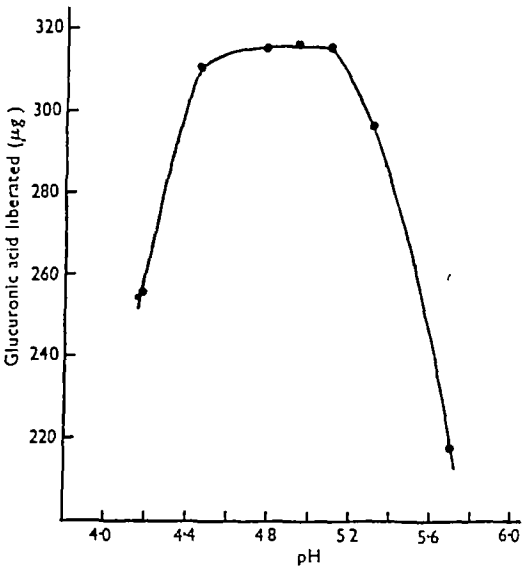


Fig 4 pH activity curve of spleen extract at stage D of preparation. Glucuronidase assays using l menthyl glucuronide.

water and dialyzed against running water for 24 hr. Fraction II was reprecipitated from the combined supernatant liquids with saturated $(\text{NH}_4)_2\text{SO}_4$ at pH 5 between 1.56 and 1.76M at 0° and the precipitate dissolved in about 200 ml of water and dialyzed against running water for 24 hr.

Stage G Stage F was repeated to obtain more pure fractions.

Data for the activity of a typical preparation at each stage of purification are presented in Table 1.

Table 1 Activity of β glucuronidase preparations at various stages of purification

Stage	Vol (ml)	Assays at pH 4.5		Assays at pH 5.0		Ratio units at pH 5.0 / pH 4.5
		Units*/ml	Total units	Units*/ml	Total units	
A	3600	3.14	11,304	2.99	10,764	0.95
B	3400	4.02	13,668	3.90	13,260	0.97
C	780	17.13	13,361	16.45	12,831	0.96
D	580	16.79	9,738	16.45	9,541	0.98
E Fraction I	35	133.12	4,659	114.61	4,011	0.86
Fraction II	220	15.91	3,502	17.85	3,927	1.12
F Fraction I	33	98.00	3,234	82.12	2,710	0.84
Fraction II	230	11.92	2,741	13.95	3,208	1.15
G Fraction I	24	111.20	2,669	90.04	2,161	0.81
Fraction II	31	72.61	2,251	85.63	2,654	1.18

* Menthyl unit of glucuronidase activity, see p 127

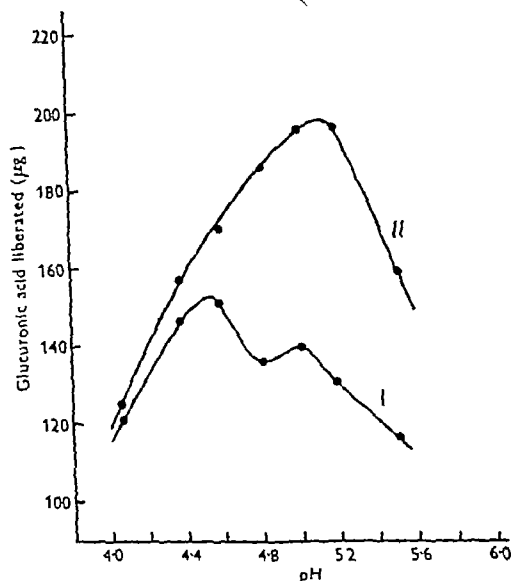


Fig 5 pH activity curves of fractions I and II at stage E of preparation. Glucuronidase assay using *l* menthyl glucuronide. I, fraction precipitated between 1.26 and 1.54M $(\text{NH}_4)_2\text{SO}_4$, II, fraction precipitated between 1.54 and 1.78M $(\text{NH}_4)_2\text{SO}_4$.

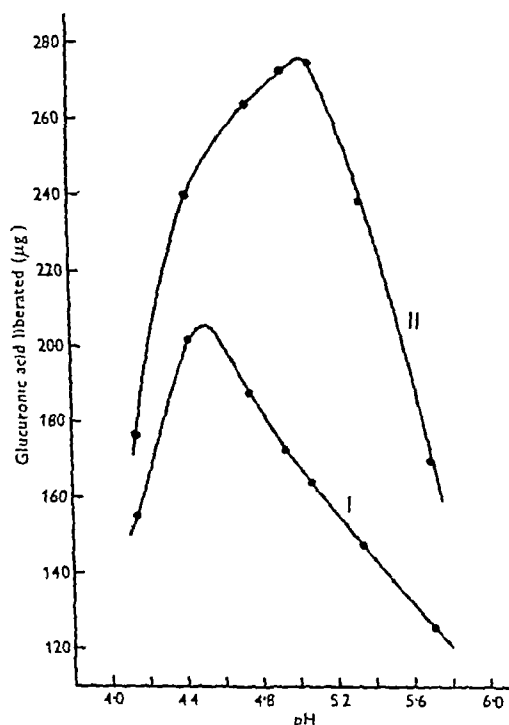


Fig 6 pH activity curves of fractions I and II at stage G of preparation. Glucuronidase assays using *l* menthyl glucuronide. Details as for Fig 5.

pH activity curves using *l* menthylglucuronide were determined for the preparations at stages D, E and G, and typical data are presented in Figs 4, 5 and 6 respectively. The pH activity curve at stage D in some preparations showed evidence of two peaks, in others, the curve shown in Fig 4 was obtained. pH activity curves for the pure fractions were also determined using phenyl and phenolphthalein glucuronides, and the data for all three substrates are shown in Table 2.

Table 2 pH optima of ox spleen β glucuronidases

Substrate	β Glucuronidase	
	I	II
<i>l</i> Menthylglucuronide	4.5	5.0
Phenylglucuronide	4.5	5.2
Phenolphthalein glucuronide	4.5	5.2

DISCUSSION

It would appear from the work of Massamune (1934), Oshima (1936), Fishman (1939 *a, b*) and Talalay *et al* (1946) that these authors considered they were dealing with a single enzyme. This conclusion may reasonably be drawn from the data they presented on the kinetics of the hydrolytic reaction. While presenting no data upon optimum pH or reaction kinetics, Graham (1946) gave no evidence which could indicate that there was more than one enzyme present in his extracts.

By the application of the variable solvent solubility test it has been possible to show the presence in spleen extracts of two proteins having β glucuronidase activity. The fact that the two enzymes have different pH optima makes extremely difficult the interpretation of the specific property solubility test of Falconer & Taylor (1946). At pH 5 the two proteins having glucuronidase activity are precipitated by ammonium sulphate within the limits 31.5–38.5% and 38.5–44% saturation, these two fractions having pH optima for the hydrolysis of *l* menthylglucuronide of 4.5 and 5.0 respectively. Fishman (1939 *a*) used the limits 37–50% saturation and Graham (1946) 36–46% saturation with ammonium sulphate in the preparation of their enzyme extracts. It is obvious, therefore, that these workers eliminated the major portion of the enzyme having optimum pH 4.5 for the hydrolysis of *l* menthyl glucuronide. It is probable, therefore, that the data of Fishman (1939 *b*) concerning pH optima and Michaelis constants may be substantially correct for one of the enzymes.

In later work Talalay *et al* (1946) used a preparation from a mixture of livers, spleens and kidneys of mice and made no attempt at fractionation. The data presented concerning the optimum pH for the hydrolysis of phenolphthalein glucuronide (4.5) and

reaction kinetics will need revision, since no account is taken of the dual nature of the spleen enzyme and any possible differences of behaviour of the liver and kidney enzymes. Further work on the reaction kinetics of these two enzymes and also upon liver and kidney glucuronidases is in progress.

SUMMARY

I. A reinvestigation of the methods for the purification of α -spleen β -glucuronidase using the variable solvent solubility test has revealed the

presence of two fractions having different pH optima. The two enzymes have been separated and purified by fractional precipitation with ammonium sulphate and shown to have the following pH optima with *l*-menthylglucuronide 4.5 and 5.0, phenylglucuronide 4.5 and 5.2, phenolphthalein glucuronide 4.5 and 5.2.

2. Methods of β glucuronidase assay using *l* menthylglucuronide, phenylglucuronide and phenolphthalein glucuronide are presented.

3. The relation of these results to those of previous workers is discussed.

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The Colorimetric Estimation of Stilboestrol, Hexoestrol and their Glucuronides in Urine

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Any attempt to estimate the synthetic oestrogens stilboestrol (4,4'-dihydroxy $\alpha\beta$ diethylstilbene) and hexoestrol or their detoxication products in urine is complicated by the presence of other phenolic substances which may give rise to interfering colours in procedures depending on colorimetry, or lead to high blank values from control urines. The dual problem presented in such work is to retain a sufficient quantitative recovery of the oestrogen while reducing blank values to a minimum. Sahasrabudhe & Wilder Smith (1947) have largely overcome this

problem in the case of dienoestrol by taking advantage of its diene structure and its reactivity with maleic anhydride. For the two more stable oestrogens, stilboestrol and hexoestrol, it appears that no satisfactory method of estimation in the presence of urine has yet been proposed, though attempts have been made to apply the Dingemans (1940) method to this problem (Dingemans & Tyslowitz, 1941; Bass & Salter, 1943). The present communication describes methods of preliminary extraction which enable estimations of these two clinically important oestrogens and of their glucuronides to be made in urine by the nitration method proposed by Malpress (1945).

* Part of the work reported in this paper was carried out at the National Institute for Research in Dairying, Shinfield, Reading.

EXPERIMENTAL

Reagents

Ether Freshly distilled ether (B P), giving no more than a faintly positive reaction for peroxide with ferrous ammonium sulphate and potassium thiocyanate, is used at all stages, possible effects of peroxide during hydrolysis are considered later in this paper

Aluminium oxide Mayfair brand (Savory and Moore Ltd) was used with no preliminary treatment

Calcium hydroxide Various samples have been used (e.g. Baird and Tatlock labelled 'from marble' or 'low in arsenic'), and there is an indication that oestrogen recoveries may vary slightly from sample to sample. It is advisable therefore to start with sufficient quantities of the purest product obtainable for a programme of work to be completed with one batch, and to carry out preliminary check recoveries of added oestrogens rather than to assume the recoveries found in this study. Coarse particles are removed by sifting through a 100 mesh sieve

Methods

In developing these methods cow's urine was used. Subsequently simplified modifications suitable for use with human urine were established. The various methods are given separately below and the processes which form the basis of the extractions are summarized in Table 1

Table 1 *Effect of reagents on free and conjugated stilboestrol and hexoestrol*

	Saturated NaHCO ₃ solution	Al ₂ O ₃	Ca(OH) ₂
Free oestrogens	Insoluble	Unadsorbed	Adsorbed
Oestrogen glucuronides	Soluble	Adsorbed	Adsorbed

In all the modifications of the method Al₂O₃ is used for the removal of pigmented and other substances, leading to a reduction of blank values

Recoveries of oestrogen, adsorbed on Ca(OH)₂ from solutions of known strength and estimated after solution in conc. HCl and extraction with ether, indicate that about half the losses associated with the methods may be ascribed to losses at this adsorption stage. This is borne out by a comparison between recoveries from cow and human urines of both the free oestrogens and their glucuronides (see Fig. 1)

Free stilboestrol and hexoestrol in cow's urine

(A) The urine is filtered and made strongly acid to Congo red with conc. HCl. A suitable portion containing 0.5–2 mg oestrogen is extracted by hand shaking in a separating funnel with 100 ml ether. Where the probable oestrogen content is not known, 100 ml urine is a convenient amount for a trial estimation. Emulsions which may form at this stage are broken by centrifugation, or, if separation is nearly complete, by the addition of anhydrous Na₂SO₄. The ether extract is shaken twice with 25 ml. saturated NaHCO₃ solution which removes the conjugated (glucuronide) forms of both oestrogens

(B) The ether extract is now shaken with 50 ml. 2N NaOH solution which removes the free oestrogen, the

alkaline solution after acidifying with 12.5 ml. conc. HCl is back-extracted with 50 ml. ether. The ethereal solution is washed once with 25 ml. saturated NaHCO₃ solution and once with 25 ml. water, dried by anhydrous Na₂SO₄ and passed with light suction through an alumina column 5 cm. high prepared in a tube of c. 1 cm. diameter. The flask and the column are washed twice with 5 ml. dry ether

(C) Finely powdered Ca(OH)₂ (2 g) is added to the ethereal solution and, after dispersion through the liquid by careful rotary hand movement for a few seconds, allowed to settle. The ether is decanted and the solid washed once with 15 ml. ether, which is also decanted, these ethereal solutions are discarded. The last traces of ether are evaporated from the Ca(OH)₂ by immersing the flask in warm water, the dry solid is suspended in 25 ml. water and dissolved in 7.5 ml. conc. HCl. The acid solution is extracted with 50 ml. ether

(D) After washing twice with 25 ml. water to free it from acid and drying with anhydrous Na₂SO₄, the ethereal solution is evaporated to small volume and quantitatively transferred to a 50 ml. volumetric flask. It is evaporated to dryness, care being taken to remove any condensate on the neck of the flask by an air current, and the residue dissolved in 6 ml. glacial acetic acid and estimated by the nitration method (Malpress, 1945)

Stilboestrol and hexoestrol monoglucuronides in cow's urine

The saturated NaHCO₃ solution from (A) or, alternatively, from a second urine sample treated as in (A), is made strongly acid to Congo red by the addition of 6 ml. conc. HCl and extracted with 50 ml. ether. The ether is dried with anhydrous Na₂SO₄ and, after decanting and washing the solid twice with 5 ml. ether, the combined extract is treated with Ca(OH)₂ as in (C) above. The ethereal solution finally resulting from this treatment is shaken twice with 25 ml. saturated NaHCO₃ solution and the combined aqueous solutions acidified strongly by the addition of 20 ml. conc. HCl (final conc. 2–2.5N). Hydrolysis is carried out by heating this acid solution under reflux for 0.5 hr

After cooling, the solution is extracted with 50 ml. ether, and the ether extract washed with 25 ml. saturated NaHCO₃ solution, then with 25 ml. water and dried with anhydrous Na₂SO₄. The solution is passed through an alumina column, as for the free oestrogens, and afterwards evaporated to small volume, transferred to a volumetric flask and prepared as outlined above for estimation

Free stilboestrol and hexoestrol in human urine

The procedure is the same as that for the estimation of free oestrogens in cow's urine except that the adsorption on Ca(OH)₂ is omitted. The ethereal solution obtained after passage through the alumina column is immediately concentrated, transferred to a volumetric flask and prepared for estimation

Stilboestrol and hexoestrol monoglucuronides in human urine

The procedure follows the corresponding method for cow's urine but omits the Ca(OH)₂ adsorption. The initial saturated NaHCO₃ extracts are acidified with 20 ml. conc. HCl and hydrolyzed for 0.5 hr. Treatment after hydrolysis is the same as for cow's urine

Estimation

The oestrogen estimations were carried out as described in an earlier paper (Malpress, 1945), except that the stilboestrol solutions were not heated after addition of HNO_3 . The final solutions were invariably slightly cloudy and were filtered through sintered glass filters, porosity G4. Filter papers were unsatisfactory, adsorbing appreciable amounts of colour.

Measurements were made with a Spekker photoelectric absorptometer using a 'spectrum violet' filter (Ilford 601, 430 $\text{m}\mu$) in conjunction with heat-absorbing filters (Hilger H503). Both control and test solutions were read against water in the second cell and with the drum reading at unity.

Hydrolysis

(a) Evidence has already been presented (Malpress, 1946) of the partial conversion of stilboestrol to the ψ (cis) isomer when it is treated with 2.5N aqueous HCl at 100° . The change is accompanied by a loss of 27.5% of the chromogenic power in the nitration reaction. Similar losses are sustained in the hydrolysis of stilboestrol glucuronide, a crystalline sample prepared by the method of Mazur & Shorr (1942) giving only 70% of the theoretical colour development after hydrolysis for 30 min with 2.5N HCl.

(b) Hydrolysis of the untreated urine results in destruction of added glucuronide either partial (hexoestrol) or complete (stilboestrol). It is essential therefore to prepare extracts as in the methods given before hydrolyzing.

(c) Traces of peroxide, if present in the ether used, are carried over to the stage of hydrolysis and greatly reduce recoveries of both oestrogens from simple aqueous solutions. Although this destruction is prevented almost completely by substances normally present in the urinary extracts submitted to hydrolysis, it would seem advisable to avoid all chance of peroxide contamination throughout the process and especially during the hydrolysis (Table 2).

Table 2 *Effect of peroxide on the recovery of oestrogens from their glucuronides after hydrolysis with 2.5N-HCl*

Extracting agent	Recovery (%)	
	Stilboestrol	Hexoestrol
Ether, peroxide free	64	87
Ether, peroxide + + + +	0	35
Ether, peroxide + + + + (urinary extract added before hydrolysis)	59	83

(d) Complete hydrolysis in 2.5N HCl is effected in 30 min with stilboestrol glucuronide, and apparently in about 2 hr with hexoestrol glucuronide (Table 3). In the latter case there is a progressive destruction of the oestrogen as hydrolysis proceeds for longer periods, indicated by a gradual yellow colouring of the solution, and accompanied by an increasing chromogenic power in the nitration reaction. This being so, it was decided to adopt 0.5 hr as the time for hydrolysis of hexoestrol glucuronide also, a point at which the hydrolysis is 90% complete and destruction negligible, as shown by the quantitative recoveries of free hexoestrol heated in acid solution with glucuronic acid for this time.

Table 3 *Effect of duration of hydrolysis in 2.5N-HCl on the recovery of oestrogens from their glucuronides*

Time (hr)	Recovery (%)	
	Stilboestrol	Hexoestrol
0.5	71	89
1	71	97
2	69	99
4	—	107
8	—	111

Preparation of glucuronides Stilboestrol monoglucuronide was isolated from rabbit's urine by the method of Mazur & Shorr (1942). It has been characterized as a dihydrate (Dodgson, Garton & Williams, 1947). Hexoestrol monoglucuronide was similarly obtained, using the ethanol ether (1:3) extraction proposed by Dodgson *et al.* (1947). The sodium salt separates readily from NaHCO_3 solution but is pigmented and attempts to remove the pigment by dissolving in water and recrystallizing after the addition of one third volume of saturated NaHCO_3 solution were not successful. Recrystallization of the acid from ether, however, gave a purified product (m.p. $179-181^\circ$). It is claimed that it is a trihydrate (Dr R. T. Williams, private communication) and the loss of weight on drying at 110° has given figures in accordance with this.

RESULTS

In most cases the individual recoveries given below represent the means of at least four separate determinations. The data on human urines, however, are from duplicates, as are all the figures for hexoestrol glucuronide recoveries. All single measurements fell well within $\pm 5\%$ of the mean values quoted.

Estimations of the free oestrogens were carried out after the addition of appropriate amounts of a 0.1% (w/v) solution of the oestrogen in glacial acetic acid to 50 or, more usually, 100 ml of distilled water or urine. The glucuronides were added in faintly alkaline aqueous solution, c. 0.1% (w/v) with respect to their oestrogen components, to the same volumes of water or urine. Invariably only single estimations were made on any one sample, the free and conjugated oestrogen recoveries being obtained from separate samples. Where human urine was used results were obtained from both mixed women's and mixed men's specimens, the recoveries being the same in both cases. All results are shown in Fig. 1.

Recovery of free and conjugated stilboestrol (Fig. 1a, b). With the process for cow's urine there is a definite increase in the percentage recovery as the original amount of stilboestrol or its glucuronide added to water is increased. Mean recoveries ranged from 66 to 79% for the free oestrogen and from 52 to 64% for the glucuronide (calculated on oestrogen content), as the weight added increased from 0.5 to 2.0 mg. This variability was not found in the mean recoveries from cow's urine which were approximately 70 and 60% respectively, for all

amounts added. The reason for this discrepancy is not clear, but since the percentage recoveries of stilboestrol from water after adsorption on $\text{Ca}(\text{OH})_2$ show a similar trend it is probable that it is introduced at this stage of the method, and may be ascribed to slight differences in adsorption characteristics due to the presence of other urinary constituents. Similar explanations may be tentatively adduced for the existence of like relationships in the recoveries of stilboestrol and hexoestrol glucuronides.

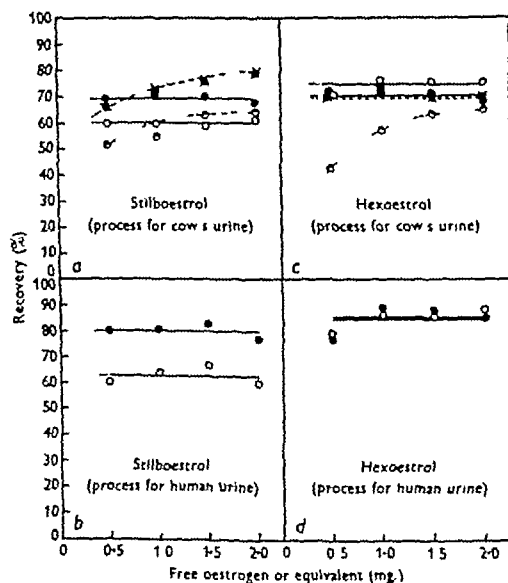


Fig. 1 Recoveries of stilboestrol, hexoestrol and their glucuronides from water and urine. \times Free oestrogen from water, \bullet Conjugated oestrogen from water, \circ Free oestrogen from urine (mean value), \circ Conjugated oestrogen from urine (mean value).

Recoveries from human urine are higher, and constant mean values of 80 and 63% for the free and conjugated forms, respectively, were obtained over the range studied. The comparatively low recoveries of the glucuronide are of course largely due to the decomposition during hydrolysis, the maximum yield being only 70–75% of the added material.

Recovery of free and conjugated hexoestrol (Fig. 1 c, d). 70% of the free oestrogen was recovered over the whole range (0.5–2.0 mg) from both water and cow's urine. The lack of agreement between the recoveries of hexoestrol glucuronide from water and from urine when the method involving lime adsorption is used has already been noted. From urine constant yields of 74% were obtained.

Mean recoveries from human urine over the complete range were equal to 85% for both the free and combined forms.

Blank values from control urines. The methods have been tested for recoveries of 0.5–2.0 mg oestrogen, and it is suggested that the initial volume of urine for extraction should be chosen to ensure an estimation in this range.

In terms of the chromogenic power of the oestrogens, blank values for the free oestrogen process using cow's urine fell normally within the range 0.05–0.2 mg oestrogen/100 ml urine. Occasionally, however, these values were greatly exceeded, blanks of 2 mg being obtained. Blank measurements for the corresponding conjugated oestrogen method were, on the other hand, invariably low and again of the order 0.05–0.2 mg oestrogen/100 ml urine.

The values given by the simplified modification applicable to human urines have been less than 0.05 mg oestrogen for the free process, and less than 0.15 mg oestrogen for the conjugated form, from 100 ml mixed urine whether from men or women.

DISCUSSION

A preliminary study of oestrogen excretion after the oral administration of a massive dose (5 g) of stilboestrol to a cow has already been communicated by Malpress & Owen (1947). During a control period satisfactorily constant blank values of 50 and 3 mg daily were obtained for the free and conjugated extraction processes in terms of oestrogen equivalents and increases in these values greater than 25 and 3 mg respectively, after administering the oestrogen, could confidently be ascribed to the effects of the treatment.

Excretion studies are to be carried out on human subjects, and it is evident from the blank values to be expected—approximately 0.5 mg for free and 1.5 mg for combined oestrogen in a 24 hr sample—that the sensitivity of the methods should be much greater when thus applied. It seems probable that excretions of the order of 0.5 mg stilboestrol or hexoestrol daily, whether free or combined, should be readily detectable, and that greater amounts should be estimated with accuracy.

The very low recoveries of conjugated oestrogen reported hitherto from the urines of experimental animals (Stroud, 1939; Dingemans & Tyslowitz, 1941; Bass & Salter, 1943) have undoubtedly been due to the failure to appreciate the degree of solubility of the glucuronides in organic solvents, and, more especially, the losses entailed when hydrolysis is performed on urine itself, rather than on suitably prepared extracts.

A recent report (Wilder Smith, 1947) claims on indirect evidence an excretion of amounts up to 6%

of the oestrogens administered to human subjects as a sulphate ester. The absence of any detectable change in the excretion of ethereal sulphate after giving the synthetic oestrogens to rabbits (Dodgson *et al* 1947) and the occurrence of only small changes of doubtful significance in the ethereal S : inorganic S ratio in the cow (Malpress & Owen, 1947) suggest, however, that these substances are normally excreted either in the free form or combined as glucuronide. If this is so the methods described in this paper should be capable of yielding comprehensive data on the quantitative excretion of the oestrogens, and afford some insight into the measure of any metabolic breakdown.

SUMMARY

1 Methods are described for the estimation of the synthetic oestrogens stilboestrol and hexoestrol and their glucuronides in cow's or human urine.

2 After addition to human urine 80% or more of these substances were recovered, except with stilboestrol glucuronide where decomposition during hydrolysis reduces the figure to 63%. Recoveries from cow's urine were slightly lower in all cases.

I wish to express my thanks to Dr R. T. Williams for samples of stilboestrol glucuronide and glucurone, and to Prof. D. C. Harrison and Dr S. J. Folley for their interest in the progress of this work.

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The Fifth Coagulation Factor ('Factor V') Preparation and Properties

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In 1943 a previously unknown coagulation factor was discovered and termed the fifth coagulation factor, or 'Factor V' (Owren, 1944). Factor V is necessary for the conversion of prothrombin to thrombin, and the rate of this reaction increases with increasing amounts of Factor V up to a certain limit. Lack of Factor V produces a haemorrhagic diathesis, termed *parahaemophilia* (Owren, 1946, 1947a). The isolation of this factor and its function in the coagulation process has been discussed in detail elsewhere (Owren, 1944, 1945, 1946, 1947b).

In the present paper experiments are described on the basis of which a new method for the preparation of Factor V has been developed, providing a more highly purified product.

METHODS

Ox plasma

Ox blood (9 vol.) was mixed with 1 vol. of 4% (w/v) potassium oxalate monohydrate solution, the mixture cooled to 1° and the plasma obtained by centrifuging at this temperature.

Prothrombin free plasma

In order to obtain Factor V absolutely free from prothrombin or thrombin, prothrombin free plasma was prepared. Prothrombin is far more readily adsorbed than Factor V by adsorbents such as $Mg(OH)_2$, $Al(OH)_3$ and $Ca_3(PO_4)_2$, but it is difficult to obtain plasma absolutely free from prothrombin with these agents. On the other hand, 10–40% of Factor V in the plasma is adsorbed simultaneously with the prothrombin depending on the agent used and the relative quantities of adsorbent and plasma (Owren, 1947b, p. 78). Asbestos differs from these agents in adsorbing prothrombin selectively. Using asbestos paper filter pads containing 30–40% asbestos, prothrombin free plasma with an almost unchanged content of Factor V can be obtained. Filters of higher asbestos content remove some of the Factor V.

The following procedure was adopted. The plasma was passed through a clarifying filter containing about 20% asbestos, and then twice through pads containing 40% asbestos (Hodgkinson, Wookey Hole, Somerset). Before filtration the pads were washed with citrate saline (0.4% (w/v) trisodium citrate, 0.9% (w/v) NaCl).

Principle *Preparation of Factor V*

Human fibrinogen prepared by the ether fractionation method of Kekwick, Mackay & Record (1946) appeared to be free from Factor V, and this encouraged an examination of the applicability of the procedure to bovine plasma.

The crude fibrinogen precipitate obtained by addition of 0.1 vol ether to plasma at unadjusted

soluble in water at pH 6.5 and above, but unlike Factor V is insoluble in acetate buffer, pH 5.23, $\mu=0.10$. Consequently it can be removed by dissolving the precipitate and reprecipitating under these conditions. In the presence of a certain amount of sodium chloride the solubility of Factor V is increased, and by this means the loss of Factor V in the precipitate is reduced to about 10%.

On the basis of these findings, the following preparative procedure was developed

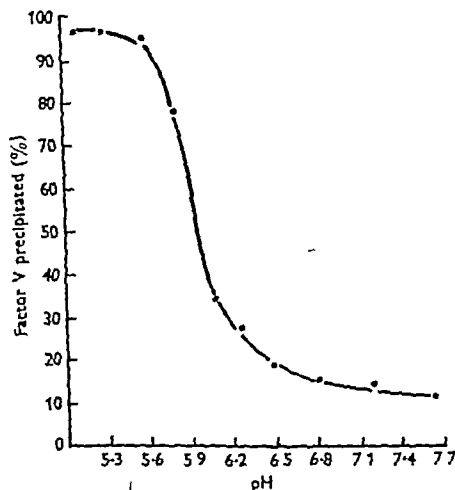


Fig 1 The precipitation of Factor V from diluted plasma with 0.1 vol ether at various pH values ($\mu=0.05$, and -1° Protein conc approx. 2%)

pH and 0° was found to contain 10% of the plasma Factor V. This was removed by repeated washing with distilled water and the fibrinogen was further purified by reprecipitation.

After the removal of the fibrinogen, about 50% of the plasma globulins were precipitated by adjusting the pH to 6.5 and lowering the ionic strength to $\mu=0.05$ by dilution with distilled water, the ether being maintained at 10% (v/v). The supernatant fluid from this precipitation contained substantially all the plasma Factor V.

The pH of samples of this fluid was further lowered and the precipitates which formed were assayed for Factor V. The precipitation of Factor V as a function of pH is shown in Fig 1. It can be seen that by lowering the pH to 5.3, the Factor V is almost completely precipitated. The amount of protein and Factor V extracted from this precipitate by acetate buffers of pH 5.23 and varying ionic strength is shown in Fig 2. From this it is obvious that by extracting the precipitate with acetate buffer, pH 5.23, $\mu=0.04$, about 30% of the protein can be removed with only a minimal loss of Factor V.

The precipitate formed at pH 5.3 contains a sticky yellow substance which like Factor V is

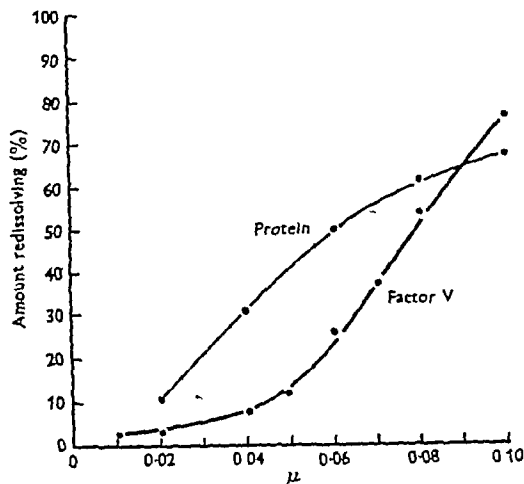


Fig 2 The solubilities of Factor V and protein (precipitated from plasma at pH 5.3 with ether) in acetate buffer at pH 5.23 and various ionic strengths

Procedure

(1) Prothrombin free ox plasma was diluted with 2 vol. of distilled water, cooled to -1° , and 10% (v/v) ether was added slowly through a capillary jet, the temperature being kept at -1 to -2° . The precipitate was allowed to settle for 3 hr and was then removed and used for the preparation of fibrinogen.

(2) The supernatant fluid was adjusted to pH 6.5 by the addition of 0.1N acetic acid and left for 2 hr at -1° . The precipitate was removed.

(3) The supernatant fluid from (2) was adjusted to pH 5.3 by further addition of 0.1N acetic acid. After 1 hr at -1° the precipitate was collected by centrifuging at -2° . This precipitate contains about 80% of Factor V in the plasma.

(4) The precipitate was washed at 0° with acetate buffer pH 5.23, $\mu=0.04$, by suspending and centrifuging. It was then suspended in a volume of distilled water equal to 20% of the original plasma volume, and dissolved by adjusting the pH to 6.5 with 0.1N NaOH. Factor V dissolved completely, and a small amount of undissolved material was removed by centrifuging.

(5) To the solution NaCl was added to give an ionic strength of $\mu=0.10$, and the pH was then lowered to 5.3 by the addition of $\frac{1}{3}$ vol. of acetate buffer, pH 5.23, $\mu=0.10$. A precipitate of yellow protein was formed and removed after 1 hr by centrifuging in the cold.

(6) Factor V was precipitated from the supernatant fluid by lowering the ionic strength to 0.04 by the addition of 150 ml distilled water to each 100 ml solution. After 2 hr at 0°, the precipitate was collected on the centrifuge, washed with distilled water and dissolved by suspending it in distilled water and adding 0.1 N NaOH to pH 7.0.

(7) The solution of Factor V was dried from the frozen state.

The yield obtained was about 60% and the purification 100–150 times in terms of activity/mg of nitrogen. It is essential that the whole procedure should be carried out at low temperature as Factor V is inactivated by ether above 0°, especially at pH 5.3.

Properties of Factor V

The dried preparation of Factor V is a white, amorphous water soluble protein material.

Stability The dried product, in sealed bottles, is stable indefinitely. When stored in solution at 0° the activity decreases about 50% in the 1st week. Higher temperatures increase the rate of inactivation. The stability on storage of Factor V in human plasma is of the same order, with a reduction to about 30% of the original activity after 8 days at 0°, and to below 10% in the same time at 10°. The rate of inactivation varies considerably. In ox plasma the stability as a rule is greater. The inactivation by heat, acid and alkali was reported upon previously (Owren, 1947b, pp. 85, 90).

Adsorption As stated above, Factor V is partly adsorbable from plasma with $Mg(OH)_2$, $Al(OH)_3$ and $Ca_3(PO_4)_2$. From purified solutions Factor V may be removed almost completely by these agents, and Seitz filtration reduces the activity considerably.

Function The influence of Factor V on thrombin formation has been described previously (Owren, 1944, 1945, 1947b, p. 182). Without Factor V no thrombin is formed.

The dried preparation of Factor V described above (0.2 mg) produced the following effects: (1) The time for complete conversion of prothrombin to thrombin in 1 ml of a mixture containing prothrombin (10 p.u.), thrombokinase (human brain, optimal amount) and Ca (2.5 mM) was shortened from ∞ to 75 sec at 37°. (2) The coagulation time for 1 ml of a mixture containing prothrombin (100 p.u.) thrombokinase (optimal amount), Ca (2.5 mM) and fibrinogen (0.10%) was shortened from ∞ to 16 sec at 37°. (3) The 'prothrombin time' by Quick's method in a case of parahaemophilia was shortened from 68 to 15 sec. The 'prothrombin time' of human oxalated plasma stored 1 week at 5° was shortened from 38 to 16 sec.

DISCUSSION

Quick (1943, 1946) has postulated the existence of two components of prothrombin, designated prothrombins A and B. Prothrombin A is labile and

disappears when plasma is stored, prothrombin B is more stable, is completely adsorbed by $Al(OH)_3$ (which does not remove prothrombin A) and its content is lowered in dicumarol poisoning.

The hypothesis is chiefly based on the fact that the prolonged 'prothrombin time' (Quick, 1943) of stored plasma is shortened by the addition of plasma, previously treated with $Al(OH)_3$ to remove adsorbable material, or plasma from animals poisoned with dicumarol.

Quick's theory has been supported by others (Onell & Lam, 1945; Zondek & Finkelstein, 1945; Munro, Hart, Munro & Walking, 1945; Munro & Munro, 1947), but questioned by Link (1945), Banfi, Bay & Tanturi (1945), Loomis & Seegers (1947). Loomis & Seegers (1947) believe that the labile component partially inactivated during storage is fibrinogen. They hold the opinion that plasma treated with $Al(OH)_3$ acts by supplying reactive fibrinogen, since the same effect could be produced with stored plasma by the addition of a fibrinogen preparation.

It has been shown previously that Factor V is the most unstable coagulation component in plasma during storage, prothrombin usually remains unchanged for 6 days in plasma stored at 0°, and the reactivity of fibrinogen keeps unaltered for at least 10 days at 0° (Owren, 1947b, pp. 84, 143, 271). The prolonged 'prothrombin time' by Quick's method in stored plasma is, therefore, first and foremost due to the inactivation of Factor V. After storage for a long time, reduced prothrombin concentration and decreased activity of fibrinogen may exert some influence. This is confirmed by the following experimental evidence.

Oxalated human plasma stored at 5° for 8 days showed a decrease of Factor V to 10% of the original value, and of prothrombin to 75%. The prothrombin time was reduced from 34 to 15 sec on addition of Factor V, whilst the addition of prothrombin (20 p.u. free of Factor V) and fibrinogen (0.10%, free of Factor V and profibrin) gave prothrombin times of 30 and 32 sec respectively.

The prothrombin time of plasma from a patient with parahaemophilia after storage for 3 weeks at 0° was reduced from 110 to 17 sec on addition of Factor V, whilst fibrinogen and prothrombin, both free from Factor V, were without influence.

The effect of fibrinogen in the experiment of Loomis & Seegers (1947) can be explained by the fact that fibrinogen precipitated from bovine plasma by the ethanol method used can be shown to contain about 10% of the plasma Factor V. Further, it should be mentioned that fibrinogen which contains profibrin will also shorten the clotting time when added to plasma.

Fantl & Nance (1946) have found that prothrombin free plasma contains a factor which accelerates

the conversion of prothrombin to thrombin, and Ware, Guest & Seegers (1947a) have isolated such a factor by fractionation of prothrombin prepared by $Mg(OH)_2$ adsorption. They suggest that this factor is not identical with any of Quick's prothrombins.

There seems to be no doubt that Quick's prothrombin A and Factor V are identical. The factor is concerned with the conversion of prothrombin to thrombin, however, and is not itself a component of prothrombin. The factor is partly adsorbed by $Mg(OH)_2$, and prothrombin prepared by this method always contains some Factor V (Prothrombin which is absolutely free of Factor V is not converted to thrombin by thrombokinase and Ca alone (Owren, 1944, 1947b, p 186)). Factor V is precipitated by the ammonium sulphate fractionation used by Ware *et al* (1947a) for isolating the accelerator factor from prothrombin preparations. The relatively slight activity of the material isolated (shortening of the conversion time to 3, 7 and 10 min) indicates that the amount of Factor V is low.

The mode of action described by Ware *et al* (1947b) for their accelerator factor, however, conforms with the previously described action of Factor V (Owren, 1944, 1945, 1947b), and there is no reason to believe that they are different substances.

The recorded properties of Factor V and experiments on a quantitative basis point to the fact that prothrombin A, Seegers's accelerator factor and Fantl & Nance's factor are identical with Factor V.

SUMMARY

1 A new method for the preparation of 'Factor V' is described, and its properties are outlined.

2 Experiments are submitted indicating that the prothrombin A of Quick (1943, 1946) and the accelerator factor of Fantl & Nance (1946) and of Ware *et al* (1947a, b) are identical with Factor V.

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The Detection of Creatine and Creatinine by Partition Chromatography

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Methods commonly used for the identification and quantitative estimation of creatinine, based on the Jaffe reaction, suffer from the lack of specificity of this reaction. Hunter (1928) has listed 40 compounds which give a positive creatinine reaction with alkaline sodium picrate. The use of 3,5-dinitrobenzoic acid (Benedict & Behre, 1936), although it provides a slightly more specific reaction, introduces the serious complications of colour fading and sensitivity of the coloured product to the presence

of other substances. The picric acid method of estimation is reliable, however, if precautions are taken to exclude the interference of other chromogenic substances, or when combined with specific enzymic destruction of creatinine by *Corynebacterium ureaefaciens* (Miller & Dubos, 1937; Krebs & Eggleston, 1939).

The estimation of creatine in the presence of creatinine by the picric acid or 3,5-dinitrobenzoic acid methods has the added disadvantage of being

a method of difference This is emphasized in the identification of small amounts of creatine in the presence of creatinine in circumstances where the Barritt diacetyl reaction for creatine (Eggleton, Elsdon & Gough, 1943) cannot be used and the Jaffé test only can be applied, since the detection must necessarily involve a quantitative determination

The technique of partition chromatography on paper, for the study of amino acids (Consden, Gordon & Martin, 1944), may be applied to the examination of inorganic and organic urinary constituents, including creatinine (Dent, 1947) The movements of these substances on paper chromatograms are sufficiently specific to permit their recognition This principle has been utilized in the qualitative identification of creatine and creatinine on the basis of differences in their physicochemical rather than chemical properties, upon which present analytical procedures rely Creatinine may be detected in biological fluids in amounts down to 1 μ g Further, creatine may be detected in the presence of creatinine without a quantitative analysis

METHODS

The experimental procedure employed was essentially that of Dent (1946), temperature control throughout experiments being maintained by placing the apparatus in a water thermostat

For the detection of creatinine, enough of the test solution to contain 5–10 μ g creatinine was pipetted in portions on to paper strips, and the water dried off The solvent was allowed to travel 25–30 cm down the strip, the time taken for the solvents collidine and *sec* butanol being c 24 hr After the strip had been dried at 100° for 30 min., creatinine was located by spraying first with 2N NaOH, then with saturated picric acid from an atomizer The resulting orange band was generally c 10 mm in width In determining R_F values (Consden *et al* 1944) it was found necessary to mark the position of the band soon after its appearance, since considerable or complete fading of the colour usually took place in sunlight

For the detection of creatine, the procedure was similar except that the strip was heated at 110° for 3 hr to convert any creatine present to creatinine Subsequent spraying of the strip with NaOH and picric acid indicated the presence of a creatine band, detected as creatinine An alternative method, described by Maw (1947) for the conversion of creatine to creatinine by spraying the strip with mineral acid and heating to dryness at 100°, has not proved entirely satisfactory, owing to the strips becoming brittle and occasionally charred The direct heating method has the advantage of simplicity, with the desirable minimum of manipulation

RESULTS

Constancy of the factors influencing the movement of a compound on a chromatogram, listed by Consden *et al* (1944), was maintained during runs Comparison of the R_F values of bands obtained from test material was made with those of creatine and

creatinine added to the test material All R_F values quoted represent the average of 6–12 determinations, deviations from mean values being at the most $\pm 5\%$ and generally not more than $\pm 2\%$

R_F values of creatine and creatinine In order to obtain a separation between creatine and creatinine, several solvents were tried, *sec*-butanol and collidine giving satisfactory results (Table 1) Creatine and creatinine showed no movement in *n* pentanol, whereas in phenol creatinine moved as fast as the solvent itself In *n* butanol the R_F value of creatine is too low to be of practical use

Table 1 R_F values of creatine and creatinine in various solvents at 17°

(20 μ l of 0.1% aqueous solution used)

Compound	Solvent		
	<i>n</i> Butanol	<i>sec</i> Butanol	Collidine
Creatine	0.04	0.18	0.19
Creatinine	0.20	0.33	0.46

Table 2 Effect of temperature on R_F values of creatine and creatinine

(20 μ l of 0.1% aqueous solution used)

Temperature	Solvent <i>sec</i> butanol		Solvent collidine	
	Creatine	Creatinine	Creatine	Creatinine
17°	0.18	0.33	0.19	0.46
20°	—	—	0.14	—
25°	0.17	0.29	0.11	0.40

A rise in temperature produced a general decrease in R_F values (Table 2) This is in part due to the influence of temperature on the composition of the solvent and water phases, and the consequent distribution of the compounds between the phases The effect of extraneous salts may be explained similarly The effect is more marked with collidine as solvent, the composition of the solvent phase changing from 41.7 to 62.8% collidine over the temperature range 10–30°, in contrast to the change for the *sec* butanol water system of 61.7 to 65.0% *sec* butanol Without temperature control, variations of 40% between values have been obtained

In Table 3 are given R_F values for creatine and creatinine determined in pure solution, in rat urine and in aqueous muscle extracts Chromatograms of rat urines gave a single band when the strips were simply dried free of solvent, and an additional slower moving band when the strips were heated at 110° Chromatograms run with added creatine and creatinine showed that the bands obtained from urine alone were attributable to these compounds The presence of urinary constituents produced distinct effects on the R_F values of the two compounds, the presence of creatinine in large excess, however,

having no effect on the movement of creatine. The solubility of creatine in water is known to be increased by the presence of urinary constituents and sodium salts as a result of salt formation. An effect on the partition coefficient of this substance between organic solvents and water would therefore be expected.

Table 3 R_F values of creatine and creatinine at 17°

(20 μ l of solution used)

	Solvent		
	n Butanol	<i>sec</i> Butanol	Collidine
Creatine			
0.1% Solution	0.04	0.18	0.19
0.025% Solution containing 0.5% creatinine	—	0.18	—
Rat urine (band I)	—	0.17	0.13
0.1% Creatine added to rat urine	0.0	0.16	0.13
5% Aqueous muscle-extract	—	0.16	—
Creatinine			
0.1% Solution	0.20	0.33	0.46
Rat urine (band II)	0.17	0.29	0.57
0.1% Creatinine added to rat urine	0.17	0.28	0.57

The bands obtained with rat urine were considerably broader than those from the pure solutions, and were much less sharply defined at the edges. Apart from the amount of material used, the width of bands is determined by the presence of salts which tend to distort the bands, either by salt formation with the compound being chromatographed, or by the production of local alterations in solvent and water-phase composition on the strip. As might be anticipated from its amphoteric nature, creatine is the more affected in this way.

R_F values have been determined on pure solutions containing 4–100 μ g of creatinine. Over this range the values were not affected by the amount of compound used. Creatine and creatinine were easily detectable in amounts down to 1 μ g.

R_F values of other 'Jaffé positive' substances. The majority of substances other than creatinine giving the Jaffé reaction may be recognized by further chemical tests, but the detection of creatinine at the same time presents numerous difficulties. These are to a large extent overcome when test solutions are examined by partition chromatography. Jaffé positive substances such as acetone, acetaldehyde, glyoxal, etc., are eliminated by volatilization during the drying of the paper strips. 'Jaffé positive' inorganic salts, e.g. ferrous sulphate, showed little or no movement in the presence of organic solvents, and were detectable only in relatively large amounts. Pyruvic and acetoacetic acids gave R_F values close

to those of creatine, but quite distinct from those of creatinine (Table 4). Since these substances give a direct Jaffé reaction, there is little likelihood of confusion with creatine. Pyruvic acid did not give bands when present in amounts much less than 100 μ g. The test was more sensitive for acetoacetic acid, but the resulting bands faded rapidly.

Table 4 R_F values of some 'Jaffé-positive' substances in pure solution at 17°

(20 μ l of solution used)

Compound	Solvent	
	<i>sec</i> Butanol	Collidine
Pyruvic acid (0.5%)	0.18	—
Acetoacetic acid (0.1%)	0.21	—
Glycocyamine (0.1%)	0.30	0.43
		(0.37 at 25°)

Glycocyamine was not converted to glycocyamine on paper strips after heating for several hours at 110°. Glycocyamine, however, gave R_F values in collidine and *sec*-butanol close to those of creatinine. No separation between creatinine and glycocyamine on chromatograms has been obtained with the solvents used, mixtures of the two compounds giving rise to one broad band. The bands obtained from equivalent amounts of the separate compounds were considerably different in their rates of colour development with alkaline sodium picrate. The creatinine band gave an intense and almost maximal colour within a few seconds. The glycocyamine band gave little or no colour in this time, and required at least 10 mm for strong colour development. The various alkyl and acyl derivatives of creatine and creatinine have not been tested.

DISCUSSION

In the identification of creatine and creatinine on the basis of partition chromatography, emphasis is transferred from the lack of specificity of the Jaffé reaction to the similarity in chromatographic behaviour of 'Jaffé positive' substances present in the test material to that of the pure compounds. It has been shown that one compound most likely to interfere with the detection of creatinine is glycocyamine, although in the amounts used the two compounds have very different rates of colour formation, which might be used to distinguish the major component of a mixture of both compounds.

The R_F values of creatine and creatinine are sufficiently far apart to give clear cut separation of the two compounds, and to enable creatine, in the presence of its anhydride, to be identified in mixtures in which the creatine content is 1% of the creatinine present or less. The limit of satisfactory detection of either substance by the Jaffé reaction is 1 μ g. With

dilute solutions, the initial pipetting of large volumes on to chromatograms may lead to the accumulation of enough salts seriously to disturb band formation and movement. For creatinine detection under these circumstances, preliminary adsorption on to Lloyd's reagent (Behre & Benedict, 1922) is suggested.

The methods described have not been extended beyond the qualitative detection of creatine and creatinine. Preliminary experiments indicate that it is doubtful whether the direct application of the Jaffé reaction to paper chromatograms can be made sufficiently quantitative, owing to the variations obtained in the width of bands and the difficulty of spraying on standard amounts of the reagents. However, the bands of both compounds, in the particular solvents used, are discrete enough for their positions to be calculated from R_f values which have been determined from duplicate experi-

ments, the bands cut out of the strips and the creatinine extracted from the paper and estimated photocolometrically. This principle has been applied with success by Flood, Hirst & Jones (1947) in the chromatographic identification and estimation of sugars.

SUMMARY

1 A method is described for the identification of creatine and creatinine in biological material in amounts down to $1 \mu\text{g}$.

2 Creatine may be detected in the presence of large amounts of creatinine without resort to a quantitative estimation.

3 The effects of various factors influencing the reproducibility of R_f values are discussed.

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The Fate of Ingested Creatinine in the Rat

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The published observations on the excretion of ingested creatinine point to a large and unaccounted loss of this substance when it is administered to rat, dog and man, generally not more than 80% of a given dose being excreted in the urine (Hunter, 1928; Wang, 1939; Beard, 1943). Since there appear to be no concomitant changes in blood urea (Fohn & Denis, 1912) or in the output of urinary urea and ammonia (Rose & Dimmitt, 1916), the fate of the unrecovered fraction of the dose has been the subject of some speculation. Claims have been made that creatinine retention takes place, or that conversion to creatine occurs (Beard & Jacob, 1939; Beard, 1943). That losses might result from bacterial decomposition in the gut has been suggested (Beard, 1943). In the earlier studies there is no evidence that intestinal absorption of an oral dose was complete and the likelihood of loss of part of a dose by

excretion in the faeces does not seem to have been considered. Dominguez & Pomerene (1945) found that in a human subject with an ileal fistula, 75% of an oral dose was recovered from the urine, in agreement with other published data on man, but, in addition, that intestinal absorption was incomplete, creatinine being recovered in the material from the fistula to the extent of 25%, sufficient to account for the apparent 'loss' of creatinine referred to by previous workers.

A study has therefore been made of creatinine absorption in the rat in order to determine whether partial recovery of ingested doses may again be explained on the basis of incomplete intestinal absorption. A series of *in vitro* experiments has also been carried out to examine the possibility of bacterial destruction of creatinine in the gut.

METHODS

Biological Adult albino rats of the Wistar strain, ranging in weight from 200 to 300 g, were used. Several of the animals were caecectomized 10 weeks prior to the experiment. The rats were housed in pairs in metabolism cages with unrestricted access to powdered rat cake and water. Urine and faeces were both collected under toluene over 24 hr periods. The type of urine/faeces separator employed (supplied by Laboratory Glass Blowers, 63 Lowlands Road, Harrow, Middlesex), which is in general use in these laboratories, is shown in Fig 1. The upper flared end is supported in contact with the base of a 10 in diam glass funnel. Faeces fall through into a collecting flask and urine runs down the funnel into the circular trough in the

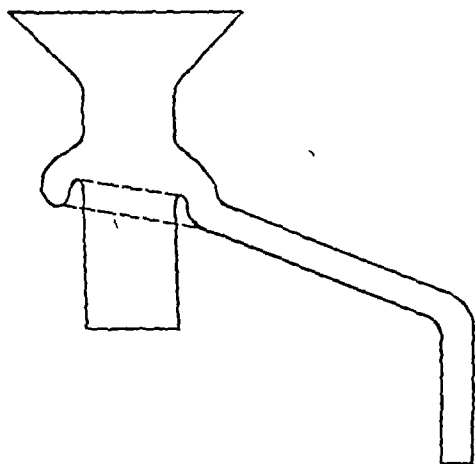


Fig 1 Urine/faeces separator (side view)

separator, whence it is drained off through the side arm. At the end of each 24 hr period each collecting funnel and separator was rinsed with water and NH_4SO_4 . For the determination of creatinine alone, the urine and rinsings were made up to 500 ml. and filtered. When creatine and creatinine were being determined, the urine and rinsings were made up to 50 ml. and filtered. The filtrate was diluted 10 times for estimations of preformed creatinine, and the undiluted filtrate used for total creatine + creatinine determinations, the analytical procedure employed giving a final tenfold dilution. The 24 hr collections of faeces were freed from excess toluene by exposing them to the air on filter papers for 15 min. They were then crushed to a thin smooth paste with a little water and the volume made up to 250 ml. The suspension was shaken for 5 min and allowed to stand for 15 min. Portions of the supernatant fluid were centrifuged at 5000 r.p.m. for 30 min and used for creatinine determinations.

Chemical Creatinine was determined on 2 ml samples of diluted urine filtrates and faecal extracts and creatine determined on 5 ml. samples of urine filtrates by methods described by Vaw (1947). Blank determinations were simultaneously carried out to compensate for the colour of the faecal extracts and for the slight increase in pigmentation of urines occurring during the estimation of creatine. Creatinine in faecal extracts was estimated enzymically

by the method of Miller, Allinson & Baker (1939) for creatinine in blood and urine. The suspensions of creatinine assimilating *Corynebacterium ureofaciens* were prepared according to the procedure of Dubos & Miller (1937), the bacteria, obtained from soil samples, being subcultured for 10 weeks on a nutrient solution containing 0.5% creatinine. Creatinine present in faecal extracts was identified chromatographically on paper strips with water saturated collidine and *sec* butanol as solvents (Vaw, 1948).

RESULTS

Oral administration of creatinine to normal and caecectomized rats The validity of a study of the fate of ingested creatinine depends on (a) accurate measurement of both urinary creatinine excreted in excess of normal amounts and creatinine excreted in the faeces in excess of substances normally present

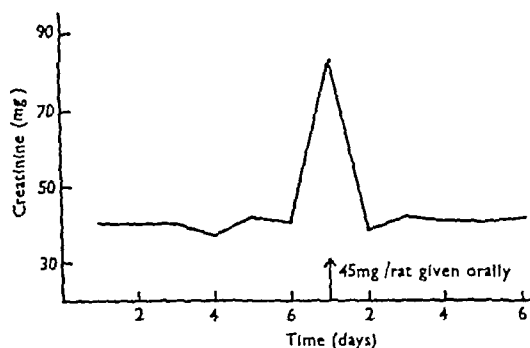


Fig 2 24 hr urinary creatinine excretion of pairs of rats given creatinine orally

giving the Jaffé reaction, and (b) avoidance of contamination of faeces by urine. To ensure (a), pairs of rats were used and were given a preliminary period of 10–14 days in the metabolism cages to accustom them to the experimental conditions. At the end of this period outputs of urine and faeces had become quite steady. Urine and faeces were then collected every 24 hr for the next 6 days, the dose of creatinine given by stomach tube in 1.5 ml water, and the urine and faeces collected daily for a further 6 days.

A typical example of urinary creatinine excretion, before and after the oral administration of creatinine to pairs of rats, is shown in Fig 2. During the first 6 day control period the 24 hr outputs showed relatively little variation ($\pm 5-7\%$), urinary creatinine rising immediately after administration of the dose and returning to predosage levels by the next 24 hr period. In order to minimize the effect of a possible variation in normal output on the day of administration, the difference between the total creatinine outputs for the first and second 6 day periods was taken as being due to the oral dose.

The excretion of substances in the faeces giving the Jaffé test (termed faecal *J+* material) showed a similar pattern over the experimental period. The 24 hr outputs from different pairs of rats, expressed as creatinine, were 2–5 mg/rat, and for a given pair of rats showed a maximum variation of $\pm 17\%$ from mean values. Forty analyses of faeces from one pair of rats gave values of *J+* material, expressed as creatinine, ranging from 0.20 to 0.26 mg/g fresh faeces.

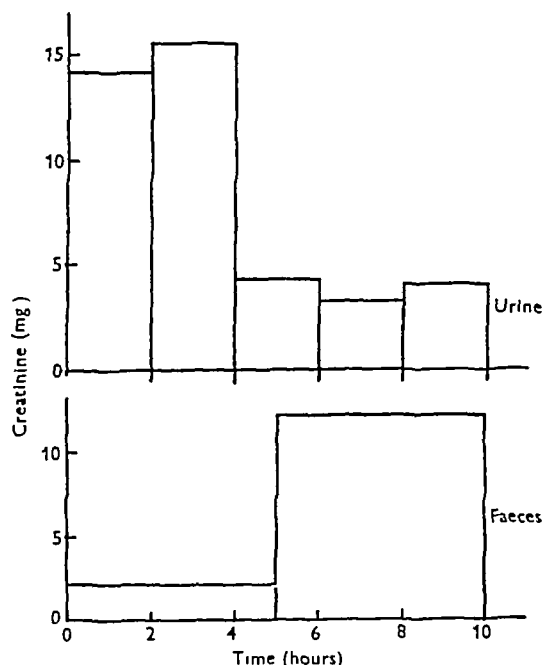


Fig 3 Urinary and faecal excretion of creatinine in two caecotomized rats following an oral dose (25 mg/rat)

The possibility of contamination of faeces by urine was checked. The type of urine/faeces separator used in these experiments was very reliable in preventing such contamination. Sixty-seven routine analyses of faeces collected from the metabolism cages gave values for *J+* material/g agreeing with those obtained from faeces collected directly from rats during defaecation. This was verified under experimental conditions by following the course of excretion of creatinine in pairs of rats for 10 hr.

immediately following its oral administration. A typical result for two caecotomized rats is given in Fig 3. Changes in faecal *J+* material occurred independently of changes in the level of urinary creatinine. The peak urinary excretion was reached within the first 4 hr, and had fallen to predose levels after 6 hr. The increases in faecal *J+* material occurred only within the second 5 hr period, and during the first 5 hr, when contamination by urinary creatinine would have been most likely, the amount/g faeces remained the same as that of faeces collected with precautions against any contact with urine. No noticeable effect on excretory levels was produced by control doses of water.

The *J+* material normally present in rat faeces has not been identified, but the rise in faecal *J+* substances resulting from an oral dose of creatinine appears to have been due to this compound. This seems certain for the following reasons:

(i) *Behaviour of the faecal 'creatinine' in the presence of creatinine-destroying enzyme suspensions from Corynebacterium ureafaciens.* The use of the enzymic method directly on faecal suspensions or extracts was not wholly satisfactory on account of the presence of enzyme-inhibiting substances in rat faeces. Creatinine solutions which were destroyed to the extent of 95% by the enzyme preparation were only destroyed to the extent of 42–49% in the same time when faeces were present. When, however, the faecal 'creatinine' at equivalent concentrations was incubated with the enzyme suspension, it was assimilated to the same extent.

(ii) *Identification of the faecal 'creatinine' by partition chromatography.* R_F values of creatinine added to faeces were compared with those of faecal 'creatinine' using one-dimensional chromatograms. The values obtained for solvents collidine and *sec* butanol are shown in Table 1.

It has been pointed out (Maw, 1948) that of other Jaffé-positive substances, at least one, glycocyamine, has R_F values close to those of creatinine. However, from the rate of colour development in the presence of alkaline sodium picrate there is strong indication that the substance present in the faeces is creatinine. The fact that the amount of faecal *J+* material in excess of the normal output, when expressed as creatinine, together with the extra creatinine excreted in the urine accounted in every

Table 1 R_F values of creatinine and faecal 'creatinine' at 17°

Dose of creatinine (mg/rat)	Solvent collidine		Solvent <i>sec</i> butanol	
	Faecal 'creatinine'	Creatinine added to faeces	Faecal 'creatinine'	Creatinine added to faeces
45	0.45	0.46	0.26	0.27
45	0.46	0.46	0.27	0.28
45	0.44	0.45	—	—
60	0.45	0.46	0.27	0.27
60	0.44	0.46	0.27	0.27

case for the administered dose (as described below), is taken as additional support

Creatinine was fed to pairs of rats in doses ranging from 30 to 60 mg /rat in a further series of 12 day experiments. Both normal and caecectomized rats were used to check the possibility of destruction of creatinine by intestinal bacteria as discussed in the note below. In both these and the 10 hr experiments part of the dose appeared in the faeces. The short term experiments indicated that this was the result of incomplete intestinal absorption, since the increase in *J* + material in the faecal pellets coincided with the appearance of animal charcoal given simultaneously with the dose. The results for the

experiments with normal rats and with caecectomized rats, in which the time taken for material to pass through the gut was reduced from about 12 to 8-9 hr, indicated little or no loss by this path. In a series of *in vitro* experiments, creatinine solutions were incubated aerobically and anaerobically with crushed fresh faeces from rats under a variety of conditions to determine the extent of bacterial destruction. Under the conditions found most favourable for creatinine breakdown, there was no aerobic destruction within the first 10 hr. Anaerobically, creatinine was found to be stable in the presence of faeces for 5-7 hr, destruction being detectable after this time and practically complete

Table 2 Urinary and faecal excretion of creatinine in pairs of rats after an orally administered dose

(*n* = normal, *c* = caecectomized, * = given by subcutaneous injection)

Dose (mg /rat)	Rat	Amount of dose (%) in		Amount of dose recovered (%)
		Faeces	Urine	
40	<i>c</i> ♂	—	40.9	—
45	<i>n</i> ♀	—	48.9	—
60	<i>c</i> ♂	—	47.5	—
30	<i>c</i> ♂	44.0	47.2	91.2
30	<i>n</i> ♂	28.3	70.2	98.5
45	<i>n</i> ♂	10.1	85.4	95.5
60	<i>c</i> ♂	34.4	67.0	101.4
45*	<i>n</i> ♂	0.0	98.7	98.7

12 day experiments are given in Table 2. The recovery from the urine varied considerably, ranging from 40 to 85% of the dose. This agrees with other published data. The faecal creatinine excreted amounted to 10-44%, and together with the fraction excreted in the urine gave almost complete recovery (91-101%) of the administered dose in every case. In one experiment, urinary creatine was also determined for periods of 11 days before and after administration. The total creatine outputs for the two periods were 121.9 and 114.8 mg /pair of rats. No extra creatinuria took place as a result of the creatinine administration. Daily variations from the mean 24 hr output were much larger than in the case of creatinine, being $\pm 50\%$. A dose of creatinine given by injection produced no rise in faecal *J* + material, indicating no apparent excretion into the intestinal tract, 99% of the dose was recovered in the urine.

Note on the destruction of creatinine by intestinal bacteria. Twort & Mellanby (1912) reported that several commonly occurring organisms were capable of metabolizing creatine, including a large Gram-positive anaerobic bacillus present in the intestine of the cat from the duodenum to the rectum, and a Gram-negative colon bacillus present in human faeces. They pointed out that the possibility of 'retention' of creatine in ingestion experiments being partly due to bacterial action could not be excluded. This might also apply in the case of creatinine administration, although the recovery

after 20-30 hr. The destruction in the presence of complete intestinal contents of rats under conditions most favourable for bacterial action was very much slower, *c.* 25% in 20-25 hr. The period during which creatinine was found not to be destroyed in the presence of faeces is of the same order as the time it would be present in the gut of a rat. Although no more than a qualitative comparison may be drawn between the *in vivo* and *in vitro* experiments, the latter suggest that any breakdown occurring during ingestion experiments is not extensive, as borne out by the recovery values.

DISCUSSION

The conversion of creatinine to creatine *in vivo* has been claimed on the grounds that creatinine ingestion or injection have been shown to give rise to temporary increases in muscle creatine in rabbits (Myers & Fine, 1913), rats (Beard & Jacob, 1939) and chicks (Almquist, Mecchi & Kratzer, 1941), with occasionally an accompanying creatinuria (Towles & Voegtlin, 1912; Beard & Jacob, 1939). However, none of the studies in which the creatinine was given orally included an examination of faeces for the compound, and no proof was provided that intestinal absorption was complete. That ingested creatinine was only partly absorbed from the small intestine of the human subject of Dominguez & Pomeroy (1945) suggests that it is unnecessary to postulate any metabolic transformation of creatinine to account for the total quantity administered.

Furthermore, the creatinine creatine conversion could not be verified by Bloch & Schoenheimer (1939) using ^{15}N -labelled creatinine fed to rats

The findings in this study on the oral administration of creatinine to rats have confirmed those of Dominguez & Pomerene (1945) on a human subject. The increased urinary excretion amounted to as much as 85 % of the dose. Creatinine was also found in the faeces, and appeared along with simultaneously administered animal charcoal. The practically quantitative recovery of administered amounts from urine and faeces together suggests that creatinine suffers little or no destruction in the body or intestine, and that the fraction of the dose which is absorbed is quantitatively excreted by the kidney.

The excretion of creatine was followed for two consecutive 11 day periods before and after creatinine administration. No increased creatinuria was observed. Beard & Jacob (1939) claim to have obtained a rise in muscle creatine of rats of 14–41 %, together with pronounced increases in creatine excretion. Beard (1943) has stated 'under normal conditions it is very easy to show a new production and excretion of excess creatine from its various precursors in the diet'. Yet in the same chapter in this monograph appears the statement 'wide variations in creatine and creatinine excretion may and usually do occur in a given individual or in different individuals'. The latter is the general finding, and deprives the former statement of its validity. The daily output of creatine is subject to considerable fluctuation, making a creatinuria due to experimental causes difficult to evaluate with any precision over short periods of time.

The fate of ingested creatinine can be adequately accounted for on the basis of its partial absorption and immediate and complete excretion, without introducing the assumption that part of a dose is retained, hydrated or otherwise metabolized. Until further information is available, another explanation must be sought for the changes in muscle creatine which have been reported. Injected creatinine has

been shown to undergo complete excretion via the kidney only. This is in agreement with the studies of Dominguez, Goldblatt & Pomerene (1935) on dogs, and those of Dominguez & Pomerene (1945) on humans, in contrast to the findings of Beard (1943). This result is in accordance with the ingestion experiments, again indicating that creatinine entering the blood stream is entirely eliminated.

Qualitatively, administered creatine behaves similarly. Only a small fraction of a dose is excreted in the urine and no apparent changes in urinary urea and ammonia are produced. Some creatine enters the muscles, but not in sufficient amounts to account for the whole of the unrecovered portion. Intestinal breakdown by bacteria has been cited as probable. From the above observations on ingested creatinine it is thought that the low urinary recovery of creatine may also be due, in part at least, to its incomplete absorption.

SUMMARY

1 Creatinine administered orally to normal and to caecectomized adult rats in doses of 30–60 mg/rat was excreted in the urine in amounts up to 85 % of the dose. No increased creatinuria was induced.

2 Creatinine also appeared in the faeces, the fraction of the dose excreted in this manner together with the fraction eliminated in the urine accounting completely, within the limits of experimental error, for the administered dose.

3 Orally administered creatinine is only partly absorbed from the intestine, the portion of the dose which is absorbed being immediately and completely excreted. It is considered unnecessary to postulate any metabolism or retention of orally administered creatinine.

4 In experiments *in vivo* and *in vitro*, creatinine has been found to suffer little or no bacterial destruction in the intestine.

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The Effect of Fertilizers on the Levels of Nitrogen, Phosphorus, Protease, and Pectase in Healthy Tobacco Leaves

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Boiled or dried leaves have generally been used in obtaining the data already in the literature on the effect of fertilizer treatment on nitrogen and phosphorus levels in tobacco leaves (Vickery, Pucher, Wakeman & Leavenworth, 1940, Ward, 1942) In addition, much work has been done on cured material

In the work reported in this paper methods of fractionation were such that sap and fibre fractions were obtained from fresh material Nitrogen and phosphorus determinations were made on all fractions, and, in addition, the levels of protease and pectase were determined on some.

METHODS

Growing of the plants Healthy tobacco (*Nicotiana tabacum* var White Burley) was grown in pots in a heated glasshouse by the Plant Pathology Department Potting material (1 kg/pot) was made up of 50% soil, 25% sand and 25% peat Analyses of the mixture indicated that it was relatively deficient in N and P, but not lacking in K. Supplements of fertilizer, when added either singly or in combination, were at the rate of 2.8 g $(\text{NH}_4)_2\text{SO}_4$, 0.75 g $\text{Ca}(\text{H}_2\text{PO}_4)_2$ and 1.1 g K_2SO_4 /pot The fertilizer was mixed with the soil when the plants were potted In each experiment eight groups of five pots were used The fertilizer treatment in the eight groups was nil, N, P, N and P, K, N and K, P and K, N, P and K. Table 1 gives the cultural history of the plants and the method of sampling employed

Harvesting of the plants Usually the plants of half the fertilizer groups were harvested 3 days later than the others (the former having been used as controls for similar fertilizer treatments with virus infected plants to be described in a subsequent paper) Plants were cut at soil level, adhering

soil brushed off, and each plant was weighed to the nearest 0.1 g In some experiments all the leaves were removed (Table 1), while in the others five leaves of corresponding position and age were removed from each plant Finally the five lots of leaves in the group were pooled and weighed.

Fractionation Within 1 hr of harvesting the samples from each treatment were minced in a domestic meat mincer and the sap expressed by hand through muslin pollam The residue after hand squeezing was remixed and again squeezed. The liquid obtained was termed 'crude sap' and the residue of fibrous material 'unwashed fibre' Washings from the mincer were added to the unwashed fibre, and more water added to bring the total to about five times the volume of crude sap This fibre suspension was squeezed through the cloth, and the fluid obtained called 'washings' Washings and crude sap were kept separate, the volumes taken, and the fibre (washed fibre) weighed

Crude sap was spun on a centrifuge for 15 min. at 3500 r.p.m. (1500 \times g), and the supernatant fluid decanted from the deposit of cell debris, starch and chloroplastic material. The supernatant fluid was called 'sap' and the deposit 'sap sediment'

Analyses (1) *Dry matter* Measured portions of each fraction were dried overnight at 95–100° and weighed

(2) *Nitrogen* Total N was determined by a micro Kjeldahl method using SeO_2 , CuSO_4 , K_2SO_4 (1:1:8) catalyst Non protein N was determined by analysis of the supernatant fluid obtained after the addition of an equal volume of 10% trichloroacetic acid. Non protein N estimations were made on the washings in the first experiment, but not subsequently, as the results obtained showed that the washings were equivalent to diluted sap For the purposes of calculation it was assumed that errors introduced by treating sap sediment and fibre N as protein N would not be large (Granick, 1938)

Table 1 Cultural history of the tobacco plants

Date potted	Date harvested	Sunshine (hr)	Average weight of whole plant with full fertilizer supplement (g)	Method* of sampling (leaves taken)
(1) 11 v 46	30 vi 46–2 i 47	158–167	34.1	5
(2) 31 x 45	21 ii 46–26 ii 46	196–222	92.0	5
(3) 4 ii 46	26 iv 46–2 v 46	187–195	105.2	5
(4) 21 v 46	11 vi 46–16 vi 46	290–333	144.5	All
(5) 31 vi 46	23 ix 46–26 ix 46	228–238	88.0	All

* Five largest leaves on 17 vi 46, 11 ii 46 and 16 iv 46, respectively in (1), (2) and (3) were marked, and harvested on the dates shown in the table

(3) *Phosphorus* was determined by a modification of the method of Kuttner & Lichtenstein (1932)

(4) *Pectase* was determined on sap and on washed fibre (Na_2HPO_4 extract, pH 8) by the method previously described (Holden, 1946)

(5) *Protease* was determined on sap and washed fibre by the method previously described (Tracey, 1948)

Analysis of data The data obtained were analyzed statistically by the methods described by Yates (1937) The results of this analysis are given in the form of main effects of N, P and K, and the interaction of N and P, other interactions being negligible The treatment effect is one quarter of the difference between the sum of the values of the four sets receiving a treatment and the sum of the values of the four not receiving it, e.g. N effect = $\frac{1}{4}(\text{N} + \text{N} + \text{P} + \text{N} + \text{K} + \text{N}, \text{P} \text{ and } \text{K}) - \frac{1}{4}(\text{O} + \text{P} + \text{P} \text{ and } \text{K} + \text{K})$ Thus if half the treatment effect is added or subtracted, according to sign, to or from the mean of all the treatments, the value for the leaves with that supplement is obtained The standard errors given are for the treatment effects Treatment effects greater than twice their standard

error are regarded as significant Extreme ranges and mean values of all treatments combined, and the means for no treatment group and N, P and K group separately, are tabulated In all, 200 plants were harvested giving 40 results for groups of five plants Figures for the two methods of sampling have not been separated This affects the values quoted for total dry matter, N and P, but does not alter the significance of the fertilizer effect on these values.

RESULTS

Dry matter, nitrogen and phosphorus The results obtained are given in Tables 2-4

Protease The results are given in Table 5 For the purpose of calculating the total protease of the leaves, it was assumed that the activity/g protein N of the washings was the same as that of the sap, and that the sap sediment fraction had no activity The figures for total protease/g total protein N indicate

Table 2 Variation in dry-matter content of tobacco plants

	Extreme range	Mean of all treatments	Mean of untreated group	Mean of N, P and K group	Fertilizer effects				Standard error
					N	P	K	N and P	
Wet weight of whole plants (g)	35.2-722.4	204.49	99.8	445.4	+98	+185	+35	+84	± 21.9
Dry matter (% of wet weight of leaves)	7.1-16.8	11.9	11.7	12.2	-0.9	+1.3	+0.1	+0.2	± 0.33
Total dry matter (g)	1.80-64.50	17.22	10.12	31.85	+6.37	+12.85	+1.22	+6.06	± 2.21
Dry matter (mg/ml sap)	31.2-71.1	50.3	50.3	54.1	+3.0	+0.05	-0.6	+3.5	± 1.3
Fibre dry matter (% total dry matter)	42-65	51.2	49.6	51.4	-2.9	+4.8	+0.5	-0.2	± 1.32

Table 3 Variation in nitrogen content of tobacco plants

	Extreme range	Mean of all treatments	Mean of untreated group	Mean of N, P and K group	Fertilizer effects				Standard error
					N	P	K	N and P	
Total N (% of dry matter of leaves)	1.03-6.89	3.25	2.85	3.22	+1.90	-0.94	-0.35	-0.22	± 0.22
Total N (mg)	75-2048	475	244.6	821.6	+425	+203	-45	+165	± 66
N (mg/ml sap)	0.94-4.52	2.20	1.86	2.23	+1.42	-0.82	-0.22	-0.34	± 0.51
Protein N (mg/ml sap)	0.47-2.62	1.20	1.04	1.56	+0.63	-0.08	-0.03	+0.16	± 0.16
Sap N (% of sap dry matter)	1.84-9.23	4.41	3.61	4.29	+2.72	-1.60	-0.31	-0.88	± 0.24
'Sap sediment' N (% of non fibre N)	9.9-30.3	17.9	17.0	15.2	-3.0	+0.3	-0.5	+1.1*	± 1.23
Sap protein N (% of sap N)	33-82	57.2	57.6	69.2	-4.6	+11.8	+2.3	+11.1	± 1.95
Fibre N (% of total N)	29-59	42.1	42.4	45.0	-3.8	+9.2	+0.9	+3.3	± 1.55
Fibre N (% of fibre dry matter)	0.74-5.65	2.65	2.41	2.77	+1.35	-0.37	-0.33	+0.17	± 0.19

* Appears to be a negative P and K effect of 2.9

Table 4 *Variation in phosphorus content of tobacco plants*

	Extreme range	Mean of all treatments	Mean of untreated group	Mean of N, P and K group	Fertilizer effects				Standard error
					N	P	K	N and P	
Total P (% dry matter)	0.09-0.52	0.245	0.14	0.34	+0.015	+0.204	-0.022	+0.080	±0.018
Total P (mg)	2.5-187.2	42.9	14.1	89.1	+18.8	+55.3	-1.8	+18.0	±8.2
Sap P (% sap dry matter)	0.09-1.09	0.331	0.14	0.47	-0.046	+0.361	-0.045	-0.049	±0.041
Fibre P (% total P)	26-58	42.1	48	39	+3.0	-6.5	-3.6	+4.4	±1.95
Fibre P (% fibre dry matter)	0.08-0.40	0.189	0.14	0.23	+0.039	+0.112	+0.043	+0.026	±0.0124

Table 5 *Variation in protease content of tobacco plants*

	Extreme range	Mean of all treatments	Mean of untreated group	Mean of N, P and K group	Fertilizer effects				Standard error
					N	P	K	N and P	
Total protease (units/g dry matter)	0.6-8.9	3.63	2.90	4.46	-0.30	+1.09	+0.36	+0.28	±0.30
Protease (units/g total protein N)	20-428	157	126.8	172	-86	+71	+25	+45	±18.9
Protease (units/ml sap)	0.03-0.63	0.30	0.23	0.37	-0.097	+0.093	+0.059	-0.013	±0.031
Protease (units/g protein N in sap)	25-1040	310	209	255	-261	+124	+86	-125	±51
Protease (% in sap)	24-86	59	57.4	56.6	-8.4	-3.0	+3.1	-5.5	±3.7
Protease (units/g dry fibre)	0.8-12.4	3.00	2.34	4.18	+0.74	+1.12	-0.05	+0.72	±0.42
Protease (units/g fibre N)	22-262	117.5	98.2	145.8	-38	+57	+15	+0.9	±11.7

Table 6 *Variation in pectase content of tobacco plants*

	Extreme range	Mean of all treatments	Mean of untreated group	Mean of N, P and K group	Fertilizer effects				Standard error
					N	P	K	N and P	
Fibre pectase (units/g dry matter)	0.066-1.31	0.35	0.40	0.23	+0.17	-0.35	-0.01	-0.07	±0.06
Fibre pectase (units/g N)	4.4-33.4	12.6	17.5	8.4	-1.1	-8.2	+1.3	+0.8	±1.7

the relative richness of the leaves in protease, and show that if all the leaf protein were hydrolyzed at the same rate as gelatin it would be completely destroyed by the leaf protease in 2-10 days (depending on the fertilizer treatment) at 40°.

Pectase The effect of fertilizer treatment on the pectase content of tobacco leaves was mentioned briefly in a previous paper (Holden, 1946). In the present series of observations pectase was determined in sap and fibre. The results (Table 6) given do not include those for sap, because in every instance the values were low, and not more than 10% of the total pectase was present in the sap. Thus the earlier observation that the application of N and P together increased the percentage of sap

soluble enzyme was not confirmed. The fibre pectase is expressed as units/g dry matter and units/g N of fibre (Holden, 1946). The results of one experiment are not included as several values were missing.

DISCUSSION

The effects found are summarized in Table 7. In spite of seasonal variations in the size and composition of the plants it was found that significant fertilizer effects were obtained from the pooled data of all experiments. While the seasonal effects are of undoubted importance it was felt that the results obtained were not sufficient for discussion in this paper.

Table 7 *Summary of effects of fertilizers on tobacco plants*

Character	Change
Wet weight	Increased by both N and P
Total dry matter	
Total N	
Total P	
Fibre P as % fibre dry matter	Decreased by N Increased by P
Dry matter as % wet weight	
Fibre dry matter as % total dry matter	
Fibre N as % of total N	
Protein N as % of total sap N	
Protease/g protein N of sap	
Protease/ml sap	
Protease/g total protein N	
Protease/g fibre N	Increased by N Decreased by P
Total N as % dry matter	
Sap N as % dry matter of sap	
Pectase/g dry matter of fibre	Increased by N
Sap dry matter/ml	
Fibre N as % dry matter of fibre	
Total N/ml. sap	
Protein N/ml. sap	Increased by P
Total P as % dry matter	
Sap P as % dry matter of sap	
Total protease/g dry matter	
Protease/g dry matter of fibre	Decreased by P
Pectase/g N of fibre	
Fibre as % total P	Decreased by N
'Sap sediment' N as % non fibre N	
Sap protease as % total protease	

The obvious effects of both N and P supplements in making the plant grow to a greater total size explain their joint action in increasing wet weight, total N, and total P. In respect of many effects, N and P appear to act in opposition. This will be seen by inspection of the second section of Table 7 to be due to P increasing the proportion of fibre, mainly carbohydrate in nature, while N increases the proportion of protein and other nitrogenous compounds. The proportion of the total N that is soluble is increased by N as fertilizer, and similarly P increases the proportion of total P that is soluble. These results might be expected. The effects of N and P on enzyme levels are, however, of more interest. The increase of protein due to N seems to be coupled with a decrease in the enzyme responsible for its breakdown, while the increase of 'fibre' (presumably involving an increase in pectin) due to P is coupled with a decrease in the enzyme responsible for beginning the breakdown of part of the fibre. Unfortunately, the rigid interpretation of the results of experiments such as these is difficult, since the two fertilizers N and P affect the levels of all quantities measured. Consequently there is no constituent to which changes can be referred. Total dry matter and total protein N have been used in the calculation of protease levels. As the total dry matter may contain from 6 to 40 % of its weight as nitrogenous compounds (if the conventional factor

of 5.8 is used for conversion) and the protein level is obviously influenced by N as fertilizer, comparison of effects is difficult. The difficulty may be put in another way: both protein and carbohydrate may be laid down in excess of the normal requirements (whatever these are), and there is no way of determining the size of these 'stores' or, alternatively, of deciding whether too little of these compounds is present for 'health'. The amount of a substance present in a plant may be regarded as a reflexion of the balance between its synthesis and breakdown. The changes in enzyme levels described are compatible with the view that these enzymes, which have a destructive action *in vitro*, are not capable of synthesis *in vivo*, by reversal of their action, and that other synthetic systems are involved.

No significant effects of potassium were observed, presumably because the K content of the soil was such that plants without K supplement were not deficient.

SUMMARY

1. The effect of supplements of nitrogen and phosphorus on the nitrogen and phosphorus content of leaf fractions of healthy pot grown tobacco has been determined. Experiments were carried out in five sets, each including eight different fertilizer treatments on groups of five plants.

2 The effect of nitrogen and phosphorus on the levels of two enzymes, pectase and protease, was also determined on the same material

3 Phosphorus increases the non-protein, or carbohydrate, components of the leaf, while nitrogen increases the protein components

4 Nitrogen increases pectase levels, while phosphorus decreases them

The reverse is true for protease. It is suggested that this may indicate the presence of different paths for the synthesis and breakdown of both pectin and protein

We wish to thank the Agricultural Research Council for grants and Mr F J Anscombe for advice on the statistical analysis

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The Effect of Infection with Tobacco-Mosaic Virus on the Levels of Nitrogen, Phosphorus, Protease, and Pectase in Tobacco Leaves and on their Response to Fertilizers

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The effect of fertilizers on the levels of nitrogen, phosphorus, protease and pectase in healthy tobacco leaves has been described in a previous paper (Holden & Tracey, 1948). The plants used in getting the data given in this paper were a precisely similar series, but were infected with tobacco mosaic virus

Bawden (1943) and Wynd (1943) have reviewed the effects of virus infection on the metabolism of plants, and references to earlier work on tobacco infected with tobacco mosaic virus can be found in their papers. The analyses described were made on tobacco plants supplied by Mr F C Bawden and Mr B Kassanis, who were studying the effects of various fertilizer treatments on the susceptibility to infection and the multiplication of tobacco mosaic virus. The data presented are restricted to a comparison of the nitrogen, phosphorus, protease and pectase levels in infected and healthy plants with different fertilizer treatments and the response to these treatments

The fertilizer treatments and levels were as described in the previous paper (Holden & Tracey, 1948)

Infection of the plants with tobacco mosaic virus In three experiments the plants were infected by rubbing five leaves with a virus preparation. These plants were harvested after 10-14 days, by which time only local virus multiplication had occurred. In the other two experiments the plants were infected when much younger, and grown for a period sufficient for the virus to spread systemically throughout the plant. The cultural history of the plants is given in Table 1

Fractionation and analyses The preparation of leaf fractions and their analysis has been described in the previous paper. pH determinations were made on spun sap using a glass electrode. Estimations of virus concentration were made on spun sap and washed fibre by Bawden & Kassanis (unpublished)

Analysis of data The initial stages of the statistical analysis of the data were as described previously, except that the results from local and systemic infection were kept separate. The results for healthy controls were also analyzed, after these had been separated into groups corresponding to controls for local and systemic infection to eliminate seasonal differences. Standard errors for the means, and for the differences between means of healthy and infected plants, were calculated for both groups (systemic and local with their corresponding controls). A pooled standard error for the difference between fertilizer effects is given, as the individual standard errors were very similar

METHODS

Plants used Tobacco (*Nicotiana tabacum* var White Burley) plants were grown in pots in a heated glasshouse

Table 1 *Cultural history of the tobacco plants*

Date potted	Date of infection	Length of infection (days)	Average weight of plant* with full fertilizer supplement (g)		Method of sampling (leaves taken)
11 x. 46	17 xu 46	14	I	33.3	5
			H	34.1	
31 x 45	12 ii 46	10	I	106.0	5
			H	92.0	
4 ii 46	17 iv 46	10	I	52.6	5
			H	105.2	
21 v 46	11 vi 46	30	I	112.2	All
			H	144.5	
31 vii 46	20 viii 46	35	I	72.4	All
			H	88.0	

* Here and in later tables, I = infected, H = healthy

RESULTS

The results obtained are given in Tables 2-6. In Table 3 relating to N, the N contents have been given both in terms of the total N, and also total N with virus N subtracted for the systemic experiments. Virus N figures were calculated from esti-

mated amounts of virus on the assumption that its N content was 16.5%.

The effects of systemic infection are summarized in Table 7.

The sap pH values are not recorded as there were no consistent differences either with different fertilizer treatments or with virus infection.

Table 2 *Dry matter content of tobacco plants*

Character determined	Con- dition of plant*	Extreme range	Mean	Standard error of difference between means of I and H†	Mean of un- treated group	Mean of N, P and K group	Fertilizer effects				Standard error of difference between fertilizer effects on I and H†
							N	P	K	N and P	
Wet weight of plants (g)	L	22-424	137	±16.1	75	285	+30	+150	+14	+55	±32.3
	H	35-526	162		75	355	+54	+162	+40	+51	
	S	20-561	192	±19.6	78	462	+137	+188	+46	+109	±39.4
	H	62-722	269		136	581	+164	+220	+26	+133	
Wet weight of leaves (g)	L	14-262	78	±7.4	51	148	+18	+64	+4	+29	±14.7
	H	22-268	79		51	147	+21	+57	+14	+22	
	S	17-412	156	±8.9	70	348	+99	+134	+33	+72	±16.4
	H	56-451	208		120	386	+101	+124	+14	+73	
Dry matter (% of wet weight)	L	7.3-16.9	11.5	±0.35	11.1	11.9	-0.65	+0.70	+0.22	-0.05	±0.71
	H	7.1-16.8	11.1		10.6	11.8	-0.57	+0.77	+0.43	+0.25	
	S	9.6-13.9	11.7	±0.42	10.9	11.6	-0.31	+1.59	-0.11	-0.59	±0.85
	H	10.0-16.1	13.1		13.3	12.8	-1.43	+2.13	-0.48	+0.13	
Total dry matter (g)	L	1.5-43.9	9.8	±1.81	5.9	20.6	+2.8	+8.9	+0.9	+4.4	±3.63
	H	1.8-44.9	9.8		5.6	19.9	+3.2	+8.0	+2.1	+3.3	
	S	1.8-51.9	18.5	±2.21	8.0	40.4	+11.4	+17.3	+3.3	+8.3	±4.42
	H	6.3-64.5	28.3		16.9	49.7	+11.1	+20.1	±0	+10.3	
Fibre dry matter (% total dry matter)	L	40-60	48.5	±0.93	49	49	-1.5	+2.0	-0.5	-1.0	±1.87
	H	42-57	49.5		47	48	-3.0	+3.5	-1.0	±0	
	S	45-66	55.3	±1.13	50	54	-5.0	+9.0	+1.0	-2.0	±2.26
	H	45-65	53.0		54	56	-4.0	+8.0	-1.0	±0	

* Here and in later tables, L = locally infected, S = systemically infected

† See Table 1, footnote

Table 3 *Nitrogen content of tobacco plants*

Character measured	Condition of plant*	Extreme range	Mean	Standard error of difference between means of <i>I</i> and <i>H</i> †	Mean of untreated group	Mean of N, P and K group	Fertilizer effects				Standard error of difference between fertilizer effects on <i>I</i> and <i>H</i> †
							N	P	K	N and P	
N (% of dry matter)	<i>L</i>	1 10-5 80	3 32	±0 16	2 88	3 47	+1 84	- 0 49	-0 36	+0 05	±0 31
	<i>H</i>	1 03-5 65	3 29		3 06	3 43	+1 61	- 0 53	-0 50	+0 12	
	<i>S</i>	2 29-6 20	4 19	±0 20	3 85	4 74	+2 02	- 1 06	-0 13	+0 18	±0 40
	<i>H</i>	1 27-6 89	3 19		2 52	2 91	+2 33	- 1 57	-0 14	-0 72	
Total N (mg)	<i>L</i>	83-763	259	±59	158	499	+172	+182	- 27	+132	±117
	<i>H</i>	75-691	258		161	447	+171	+120	±0	+ 87	
	<i>S</i>	111-2050	765	±72	290	1823	+792	+619	+120	+477	±144
	<i>H</i>	231-3282	805		370	984	+807	+327	-113	+282	
Sap N (% of dry matter)	<i>L</i>	2 10-8 80	4 53	±0 21	3 64	4 70	+2 52	- 0 76	-0 31	-0 40	±0 41
	<i>H</i>	2 17-6 90	4 49		3 90	4 70	+2 41	- 1 26	-0 25	-0 50	
	<i>S</i>	3 15-8 95	5 30	±0 25	4 52	4 80	+2 43	- 1 54	-0 58	-0 71	±0 50
	<i>H</i>	1 84-9 23	4 28		3 13	3 61	+3 19	- 2 12	-0 41	-1 45	
Sap N (mg/ml.)	<i>L</i>	1 18-3 91	2 20	±0 11	1 83	2 36	+1 22	- 0 52	-0 13	-0 26	±0 23
	<i>H</i>	1 04-4 47	2 16		1 93	2 37	+1 15	- 0 62	-0 22	-0 14	
	<i>S</i>	1 48-4 24	2 82	±0 14	2 34	2 79	+1 62	- 0 79	-0 21	-0 11	±0 28
	<i>H</i>	1 16-4 52	2 27		1 72	2 01	+1 82	- 1 10	-0 24	-0 63	
Sap protein N (mg/ml.)	<i>L</i>	0 71-2 34	1 37	±0 08	1 24	1 75	+0 52	+ 0 03	-0 01	+0 13	±0 16
	<i>H</i>	0 61-2 62	1 28		1 16	1 74	+0 54	+ 0 04	-0 02	+0 18	
	<i>S</i>	1 11-2 80	1 84	±0 10	1 57	2 20	+0 89	± 0	-0 10	+0 31	±0 20
	<i>H</i>	(0 57-2 26)	1 30		1 10	1 46	+0 81	- 0 26	-0 05	+0 16)	
	<i>H</i>	0 47-1 78	1 08		0 87	1 29	+0 77	- 0 17	-0 08	+0 14	
Sap sediment N (% of non fibre N)	<i>L</i>	10 4-22 2	16 3	±1 1	16 9	16 4	-0 1	- 0 1	-1 1	+2 4	±2 3
	<i>H</i>	10 0-24 3	16 6		14 7	15 1	-1 4	+ 1 7	-1 1	+1 3	
	<i>S</i>	12 4-23 0	16 8	±1 4	18 8	13 9	-2 7	- 1 7	-1 2	+0 7	±2 8
	<i>H</i>	9 9-30 3	19 9		20 3	15 4	-5 4	- 1 7	+0 4	+0 6	
Fibre N (% of dry matter)	<i>L</i>	0 75-4 98	2 78	±0 15	2 64	2 92	+1 23	- 0 18	-0 47	+0 23	±0 31
	<i>H</i>	0 74-5 08	2 67		2 54	2 79	+1 07	- 0 07	-0 53	+0 37	
	<i>S</i>	2 07-4 90	3 53	±0 19	3 51	3 86	+1 40	- 0 69	-0 16	+0 47	±0 37
	<i>S</i>	(0 51-3 77)	2 41		2 69	2 11	+1 14	- 1 37	-0 10	+0 19)	
	<i>H</i>	1 07-6 65	2 62		2 22	2 76	+1 77	- 0 82	-0 06	-0 14	
Fibre N (% total N)	<i>L</i>	34-48	41 2	±1 26	45	41	-5 1	+ 4 3	-3 4	+2 4	±2 5
	<i>H</i>	27-50	38 8		39	39	-2 8	+ 6 2	-1 8	+2 2	
	<i>S</i>	35-57	45 6	±1 56	44	56	-1 1	+10 3	+0 1	+6 4	±3 1
	<i>H</i>	29-59	47 2		48	54	-5 4	+13 9	+0 4	+5 1	

* See Table 2, footnote

† See Table 1, footnote

DISCUSSION

The data given in tabular form offer opportunities for extended discussion and speculation. It is proposed, however, to restrict the scope of the discussion, but increase its validity, by considering only differences in means of values for infected and healthy plants, and by excluding any that are not at least twice as great as the calculated standard error for differences between means.

Local multiplication of the virus for a period of about 10 days results in no significant change in the response of the plants to fertilizer supplements, and

to a difference in composition between healthy and infected plants only in the percentage of total P that is in fibre.

Continued multiplication of the virus with its spread throughout the leaves has some effect on the response to fertilizer treatment, and causes profound changes in the size and composition of the leaves. There are four instances of significant changes in fertilizer response. The positive effect of P supplements on the total N of the leaves is increased and the K effect is reversed. The negative effect of N and the positive effect of P on the protease units/g protein N of sap are both significantly enhanced. In

Table 4 *Phosphorus content of tobacco plants*

Character measured	Condition of plant*	Extreme range	Mean	Standard error of difference between means of <i>I</i> and <i>H</i> †	Mean of untreated group	Mean of N, P and K group	Fertilizer effects				Standard error of difference between fertilizer effects on <i>I</i> and <i>H</i> †
							N	P	K	N and P	
P (% of dry matter)	<i>L</i>	0 096-0 593	0 256	±0 020	0 131	0 244	+ 0 016	+ 0 175	- 0 032	- 0 022	±0 040
	<i>H</i>	0 092-0 455	0 238		0 198	0 472	- 0 003	+ 0 216	- 0 021	- 0 008	
	<i>S</i>	0 182-0 600	0 318	±0 024	0 214	0 428	+ 0 030	+ 0 210	- 0 080	- 0 046	±0 048
	<i>H</i>	0 142-0 470	0 247		0 155	0 330	+ 0 030	+ 0 170	- 0 040	- 0 016	
Total P (mg)	<i>L</i>	5 32-66 35	25 20	±2 1	5 51	35 84	+ 7 73	+31 03	- 2 20	+ 8 21	±4 3
	<i>H</i>	2 51-36 46	24 20		6 70	43 81	+ 3 84	+28 80	+ 2 94	+ 4 06	
	<i>S</i>	4 30-165 4	65 50	±2 7	17 00	158 60	+50 84	+90 11	+ 1 64	+46 75	±5 4
	<i>H</i>	10 10-187 2	75 40		25 26	167 00	+42 24	+95 01	- 9 05	+38 95	
Sap P (% of dry matter)	<i>L</i>	0 10-0 59	0 357	±0 054	0 100	0 230	- 0 043	+ 0 236	- 0 010	- 0 070	±0 108
	<i>H</i>	0 09-0 78	0 295		0 130	0 430	- 0 038	+ 0 330	- 0 033	- 0 034	
	<i>S</i>	0 16-0 89	0 432	±0 086	0 220	0 550	- 0 070	+ 0 430	- 0 050	- 0 050	±0 133
	<i>H</i>	0 14-0 72	0 448		0 180	0 540	+ 0 066	+ 0 529	- 0 189	+ 0 054	
Fibre P (% of dry matter)	<i>L</i>	0 080-0 380	0 200	±0 013	0 130	0 221	+ 0 040	+ 0 100	- 0 050	- 0 010	±0 026
	<i>H</i>	0 080-0 400	0 183		0 120	0 220	+ 0 029	+ 0 128	- 0 046	+ 0 018	
	<i>S</i>	0 185-0 395	0 250	±0 016	0 200	0 322	+ 0 061	+ 0 070	- 0 018	+ 0 061	±0 031
	<i>H</i>	0 140-0 390	0 198		0 160	0 241	+ 0 051	+ 0 089	- 0 039	- 0 039	
Fibre P (% total P)	<i>L</i>	32-56	42 5	±1 4	45 0	39 5	+ 0 3	- 0 6	+ 0 4	- 0 3	±2 8
	<i>H</i>	26-55	39 6		43 6	36 3	+ 1 6	- 4 6	- 0 9	+ 6 1	
	<i>S</i>	27-56	44 3	±1 8	47 0	40 5	+ 3 6	- 10 0	- 1 1	+ 2 0	±3 5
	<i>H</i>	29-63	45 9		54 5	43 0	+ 4 0	- 7 8	- 5 5	+ 3 3	

* See Table 2, footnote

† See Table 1, footnote

general, however, it may be said that the response to fertilizers of infected plants is similar to that shown by healthy plants

Infection in the early stages of growth, leading to systemic spread of virus is well known to result in a yellowing of the leaves and a stunting of the plant. This stunting is shown in Table 2 as a reduction in the wet weight of the leaves, and there is a greater proportionate reduction of total dry matter, resulting in a wetter leaf. The decrease in dry matter is accompanied by a decrease in the total P, but the decrease in P content is less than that of dry matter so there is an increase in the P as percentage of total dry matter, and as percentage of dry matter of the fibre fraction. The increase is greater than can be accounted for by virus P.

During the yellowing of the leaves in tobacco mosaic infection there may be a reduction in the chlorophyll content by as much as 60 % (Peterson & McKinney, 1938). The reduction in chlorophyll is presumably accompanied by a decrease in the amount of protein with which it is associated. Woods & Du Buy (1941) have shown that, particularly under conditions of N starvation, chromoprotein diminishes in leaves as the amount of virus increases. In the present experiments there was a significant decrease in infected leaves in the sap sediment N fraction, which contains much of the chromo-

protein. As the leaves taken from infected plants were in various physiological states, from small dark green and large yellow green to completely yellow leaves, the difference is not as great as would be expected if yellow leaves only from infected plants were compared with leaves of the same age from healthy plants.

The reduction in the carbohydrate content of tobacco leaves infected with tobacco mosaic virus found by Dunlap (1930, 1931) agrees with the findings reported here, for, while there is a decrease in the total dry matter, there is no decrease in total N. Dunlap found that there was a greater decrease in the starch and sugar content than in that of the other carbohydrates. This would be in agreement with the present findings that there was a greater loss of soluble fractions, which resulted in a greater proportion of the dry matter being in the fibre. Cordingley, Grainger, Pearsall & Wright (1934) also found a decrease in total carbohydrate, but found that the proportion of the various fractions was the same in infected and healthy plants. These different results may be due to the different methods of fractionation and estimation used.

As systemic infection has no significant effect on the total N of the leaves it leads to an increase in N as percentage of dry matter. The distribution of N between fibre and soluble fractions is not altered,

COMPOSITION OF VIRUS-INFESTED LEAVES

Table 5 *Protease content of tobacco plants*

Character measured	Con dition of plant*	Extreme range	Mean	Standard error of difference between means of <i>I</i> and <i>H</i> †	Mean of untreated group	Mean of N, P and K group	Fertilizer effects				Standard error of difference between fertilizer effects on <i>I</i> and <i>H</i> †
							N	P	K	N and P	
Total protease (units/g dry weight)	<i>L</i>	1.4-10.5	4.2		3.7	5.0	-0.7	+1.3	-0.2	-0.4	± 0.68
	<i>H</i>	2.0-8.9	4.2	± 0.34	3.3	4.9	-0.5	+1.4	+0.4	+0.1	
	<i>S</i>	1.4-7.1	3.3		2.6	4.5	+0.4	+1.5	+0.2	+0.6	± 0.85
	<i>H</i>	0.6-4.1	2.7	± 0.42	2.2	3.8	± 0.0	+0.7	+0.3	+0.6	
Protease (units/g protein N)	<i>L</i>	48-545	186		185	148	-70	+18	-49	-58	± 40
	<i>H</i>	35-428	183	± 20	136	184	-91	+74	+24	-26	
	<i>S</i>	36-225	101		110	154	-43	+56	+15	-18	± 48
	<i>H</i>	20-203	117	± 24	78	100	-55	+41	± 0.0	+25	
Protease (units/ml sap)	<i>L</i>	0.04-0.60	0.34		0.29	0.46	-0.13	+0.15	-0.01	+0.04	± 0.062
	<i>H</i>	0.07-0.63	0.34	± 0.031	0.25	0.39	-0.13	+0.08	+0.08	-0.04	
	<i>S</i>	0.07-0.42	0.22		0.17	0.26	-0.01	+0.10	+0.02	+0.01	± 0.077
	<i>H</i>	0.03-0.49	0.25	± 0.038	0.19	0.33	-0.05	+0.12	+0.03	+0.02	
Protease (units/g dry weight sap)	<i>L</i>	1.0-15.0	7.1		5.6	9.2	-2.6	+3.4	-0.4	+0.5	± 1.25
	<i>H</i>	1.5-13.3	7.1	± 0.62	5.0	7.5	-3.3	+1.4	+2.4	-1.3	
	<i>S</i>	1.2-8.8	4.3		6.7	3.0	-0.9	+2.0	+0.4	-0.5	± 1.53
	<i>H</i>	0.5-10.5	4.9	± 0.76	3.7	0.7	-1.5	+2.4	+0.5	-0.1	
Protease (units/g protein N of sap)	<i>L</i>	36-792	290		236	293	-230	+154	-16	-37	± 80.0
	<i>H</i>	38-892	316	± 39.9	213	251	-248	+140	+90	-125	
	<i>S</i>	27-350	141		112	127	-78	+67	+23	-41	± 97.2
	<i>H</i>	25-1040	300	± 48.6	204	261	-279	+224	+78	-126	
Protease (units/g dry weight of fibre)	<i>L</i>	1.0-11.7	3.7		3.4	5.1	+0.4	+0.7	+0.6	+0.6	± 0.77
	<i>H</i>	1.4-12.4	3.4	± 0.38	2.7	4.9	+0.9	+1.7	-0.3	+0.7	
	<i>S</i>	1.7-8.8	3.4		2.5	4.2	+1.0	+1.3	-0.7	+0.9	± 0.94
	<i>H</i>	1.2-3.8	2.4	± 0.47	1.8	3.6	+0.6	+0.2	+0.4	+0.7	
Fibre (units/g N)	<i>L</i>	21-284	128		121	149	-44	+37	+27	-6	± 17.3
	<i>H</i>	22-262	132	± 8.6	107	159	-34	+73	+9	-12	
	<i>S</i>	35-196	93		68	105	-16	+55	-9	+1	± 21.2
	<i>H</i>	26-150	98	± 10.6	85	126	-43	+33	+23	+20	

* See Table 2, footnote

† See Table 1, footnote

Table 6 *Pectase content of tobacco plants*

Character measured	Con dition of plant*	Extreme range	Mean	Standard error of difference between means of <i>I</i> and <i>H</i> †	Mean of untreated group	Mean of N, P and K group	Fertilizer effects				Standard error of difference between fertilizer effects on <i>I</i> and <i>H</i> †
							N	P	K	N and P	
Fibre (units/g dry matter)	<i>L</i>	0.10-0.61	0.29		0.35	0.24	+0.125	-0.234	-0.013	-0.026	± 0.10
	<i>H</i>	0.07-0.51	0.26	± 0.050	0.34	0.20	+0.106	-0.216	-0.009	-0.028	
	<i>S</i>	0.08-0.95	0.46		0.55	0.41	+0.194	-0.340	+0.081	-0.038	
	<i>H</i>	0.08-1.31	0.42		0.46	0.25	+0.225	-0.482	± 0	-0.103	
Fibre (units/g N)	<i>L</i>	4.35-22.20	11.60		15.30	11.28	-1.37	-6.50	+2.16	+2.90	± 3.00
	<i>H</i>	4.45-17.20	9.81	± 1.50	13.01	9.07	-0.16	-5.32	+1.68	+0.72	
	<i>S</i>	3.78-24.10	12.33		15.50	10.61	+1.62	-7.37	-0.72	+2.16	
	<i>H</i>	6.70-33.45	15.58		21.92	7.65	-2.25	-14.65	+0.69	+0.69	

* See Table 2, footnote

† See Table 1, footnote

‡ For definition of units see Holden (1946)

and there is a corresponding increase in N as percentage of dry matter of both sap and fibre fractions. One of the results of infection is an increase in the rate at which the leaves yellow, which leaves of healthy plants do with increased age. The yellow healthy leaves, like the infected leaves, have a higher water content than healthy green leaves (Smirnow, 1940), but, unlike infected leaves, have less N/100 g dry matter. If virus N is subtracted from the total N the infected leaves still do not have less N/100 g dry matter, so infection cannot be regarded simply as a premature ageing of the leaves.

Table 7 *Effects of systemic infection of tobacco plants*

Decrease in	Wet weight of plants
	Wet weight of leaves
	Total dry matter
	Total phosphorus
	Dry matter as % wet weight
	Sap sediment N as % non fibre N
	Protease/g protein N of sap
Increase in	Pectase/g N of fibre
	Total N as % dry matter
	Fibre N as % dry matter
	Sap N as % dry matter
	Total P as % dry matter
	Fibre P as % dry matter
	Sap N/ml
	Protein N/ml
	% total dry matter as fibre
	Total protease/g dry weight
No significant change in	Fibre protease/g dry weight
	Total N
	% total N on fibre
	% total P on fibre
	Sap P as % dry matter
	Total units protease/g total protein N
	Fibre protease/g N
	Pectase/g dry matter of fibre

Considering the mean of all treatments, about one third of the total N is virus N, in agreement with Bawden & Pirie (1946). But the range is from 10 to

65 % of the total N as virus N, and in one experiment (with plants given P but no N) 80 % of the fibre N was virus N. It is certain that the protein components of systemically infected leaves are affected differently, as, for example, the decrease in chromo protein mentioned earlier. Several authors have referred to changes in enzyme levels caused by virus infection (Balls & Martin, 1938, Peterson & McKinney, 1938, Wynd, 1942). In the present experiments systemic infection affected protease and pectase levels differently. Fibre pectase was related to non virus N, while fibre protease was related to total N levels. Sap protease, on the other hand, was related to non-virus protein rather than to total protein N.

SUMMARY

1 The effects of supplements of nitrogen and phosphorus on the nitrogen and phosphorus content of leaf fractions of tobacco plants with local and systemic multiplication of tobacco-mosaic virus were similar to those found with healthy plants.

2 The effect of local multiplication of tobacco mosaic virus on the composition of the leaf fractions studied was negligible.

3 The effect of systemic multiplication of tobacco mosaic virus on the composition of the fractions studied was profound.

4 Total nitrogen was not affected, while dry matter and phosphorus were reduced.

5 The levels of the two enzymes studied were affected differently. Fibre protease was related to total fibre nitrogen while pectase appeared to be related to non virus fibre nitrogen.

We wish to thank the Agricultural Research Council for grants and Mr F J Anscombe for advice on the statistical analysis.

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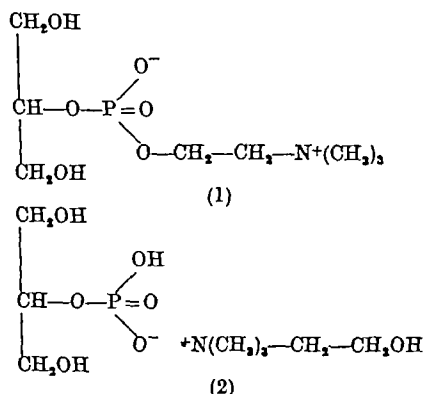
Glycerylphosphorylcholine and Choline Glycerophosphate

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In 1938-9 at the British Postgraduate Medical School, King & Aloisi isolated from dried pancreas a glycerophosphoric ester of choline which was found to be readily hydrolyzable. Evidence was also obtained for the existence of a dicholine ester. This work was published only in 1945 owing to war difficulties. In the same year Schmidt, Hershman & Thannhauser (1945) reported the isolation of α -glycerylphosphorylcholine from the same material. They also found that the ester was very rapidly split by N hydrochloric acid.

We have now tried to synthesize the choline esters of both α and β glycerophosphoric acid in order to examine their metabolic behaviour in animal tissues and other cells. The products obtained by the few methods recorded in the literature for the synthesis of β glycerylphosphorylcholine (Arnold, 1940, Ravazzoni & Fenaroli, 1940, Belfanti, Ercoli & Francioli, 1940, Mihalik, Jeney & Uri, 1941) seemed unlikely to us to be esters of choline (1), but rather to be salts of choline and β glycerophosphoric acid (i.e. β glycerophosphate of choline) (2).



After having attempted the synthesis in several ways which are briefly described, we decided to follow a method analogous to that used by Kabashima (1938) for the synthesis of lecithins (though this still requires assessment). Ravazzoni & Fenaroli (1940) also attempted this, but with no clear result. Bromocholine picrate and silver glycerophosphate were treated in hot absolute ethanol for several hours, followed by removal of picric acid and purification.

EXPERIMENTAL

Preparation of choline β glycerophosphate

Method of Arnold (1940) This consists in boiling bromocholine bromide (trimethyl β bromoethylammonium bromide) and silver β glycerophosphate in absolute ethanol. Arnold obtained a hygroscopic crystalline product, m.p. 104-105°, with a P/N atomic ratio = 1 and good analytical figures, it was precipitated from ethanolic solutions by ether and other solvents. In Arnold's paper there is no report about either a test for free choline (Florence's reaction, cf. Stanek, 1908) or the behaviour of the substance on hydrolysis. But he quoted a property of his substance the significance of which could hardly be misleading, the cadmium precipitate of his 'ester' did not contain nitrogen. These properties were sufficient to raise the question whether Arnold's product was really an ester of choline.

In our experiments we boiled equivalent amounts of silver β glycerophosphate (from the Na salt, 100% pure β isomer by HIO_4 analysis) and bromocholine bromide (Krüger & Bergell, 1903, m.p. 230°) in absolute ethanol for 7 hr in a N_2 atmosphere with thorough stirring. After removal of AgBr and several precipitations with ether, we obtained from ethanol ether mixture a fairly good crystalline product, which showed the same properties as described by Arnold. It was very hygroscopic, with m.p. 104° when thoroughly dried over P_2O_5 . (Found N, 5.15, P, 11.24, choline (method of Roman 1930), 40.3. Calc. for $\text{C}_8\text{H}_{12}\text{O}_6\text{NP}$ N, 5.09, P, 11.27, choline, 44.0%). We obtained good crystals also from ethanol acetone and ethanol pyridine mixtures (see Pl. 2, Fig. 1).

Evidence that the substance is a choline salt of β -glycerophosphoric acid (a) Ammonium reineckate gave from aqueous solutions crystals like choline reineckate which did not contain P. (b) The Florence test was positive and choline was estimated quantitatively by the Roman (1930) method. (c) With $\text{Ba}(\text{OH})_2$ the product yielded a precipitate which contained P but no N (Ba β glycerophosphate). (d) With NH_4OH in absolute ethanol at 2-4° crystals of ammonium β glycerophosphate were formed. (e) Basic lead acetate, with and without addition of NH_4OH , precipitated Pb glycerophosphate when the aqueous solution of the substance was sufficiently concentrated (4-5%). Weaker solutions gave a partial precipitate (17% from a 0.64% solution) of a mixture with P/N=1. (f) The pH titration curve showed a pK of 6.36 which corresponded to that of β glycerophosphoric acid.

Preparation of choline α glycerophosphate

This was not prepared by Arnold, but we followed the same procedure as for the β isomer. The $\text{Na}_2\alpha$ glycerophosphate from which we started contained 88% of pure α isomer (HIO_4 analysis). The yield was practically a quantitative one. The product was a light yellow hygroscopic syrup soluble in ethanol, insoluble in ether. We were not able to obtain crystals from dry ethanol-ether mixtures either after 3 months' keeping at 0° , or after 24 hr. over solid CO_2 , under these conditions the β isomer undergoes massive crystallization.

In the first steps of purification from ethanol solutions small white precipitates were formed and discarded. Finally the syrup became more and more difficult to dissolve in anhydrous cold ethanol. The P/N ratio by weight was 2.3 (theory, 2.21) after several precipitations, this showed that the substance was a well defined compound. The evidence for a salt-like nature was the same as described for the β isomer. The pH titration curve was the same as for α glycerophosphoric acid over the range studied.

Method of Mihalik et al (1941) We did not see the original paper which was published in the Proceedings of the Hungarian Physiological Society. The method was reported to consist in putting together equivalent amounts of choline base and β glycerophosphoric acid (apparently in aqueous solution), and boiling them for 36 hr. In the report it is stated that the product (m.p. $104\text{--}105^\circ$) did not contain free choline as the Florence test was negative (cf Stanek, 1908). This is a rather doubtful result, since the melting point is the same as that of Arnold's (1940) product which, as we showed above, is actually a salt. Furthermore it is impossible to understand how an ester could be yielded by the mere contact of β glycerophosphoric acid and choline in water, also in ethanol the affinity of the secondary OH group of glycerophosphoric acid for the N would be overwhelming.

We have tried to obtain the choline salt of β glycerophosphoric acid by mixing equivalent amounts of β glycerophosphoric acid and choline base (from choline chloride and wet Ag_2O , or from trimethylamine and ethylene oxide) in water, the mixture was dried at room temperature *in vacuo* over P_2O_5 , dissolved in absolute ethanol and precipitated several times with dry ether. From the ethanolic solutions of the syrupy precipitate we got beautiful crystals by adding dry ether or pyridine. The crystals, dried *in vacuo* over P_2O_5 at $70\text{--}75^\circ$ for 30 hr, were still extremely hygroscopic and showed a m.p. $93\text{--}94^\circ$. They gave the following analytical figures: Found N, 4.26; P, 9.96, P/N, 2.3. Calc. for $\text{C}_5\text{H}_{12}\text{O}_7\text{NP} \cdot \text{H}_2\text{O}$ N, 4.50, P, 9.96%, P/N, 2.2.

Inorganic P and N were absent and all the N was present as choline. Attempts to eliminate the water of crystallization were unsuccessful (over P_2O_5 at 75° *in vacuo* for 30 hr). Apart from the above-mentioned difference, the

chemical behaviour of the substance was identical with that of Arnold's product, and therefore gave further evidence of the salt-like nature of the latter.

Methods of Ravazzoni & Fenaroli (1940) These authors gave two methods for the synthesis of the choline ester of β glycerophosphoric acid. One is the same as that of Arnold, with the irrational variant of operating in water as well as in methanol. The other is based on the reaction of bromocholine picrate with monosilver glycerophosphate suspended in water. After removal of picric acid and purification, the authors obtained a crystalline product which showed good analytical figures for $\text{C}_5\text{H}_{12}\text{O}_7\text{NP}$. They did not give the melting point of the substance, but they stated that the Florence test was negative and became positive only after hydrolysis with hot HCl. No information was given about the yield.

We repeated the preparation as described by Ravazzoni & Fenaroli and did not get a crystalline product, but only a little light yellow syrup with a very low N content (P/N ratio by weight = 5), which with basic lead acetate gave a precipitate free from N. The Florence test was frankly positive, while in dilute solutions we had no evidence of choline reneckate crystals. After hydrolysis with 2N HCl at 100° the same dilute solution showed crystals of reneckate, it is well known that ammonium reneckate gives silky crystals with trimethylamine, which is, however, negative to the Florence test.

Conclusion It is obvious that none of these methods is likely to give the choline esters of glycerophosphoric acid. Actually a crystalline compound similar to that described by Arnold can be obtained simply by mixing equivalent amounts of β glycerophosphoric acid and choline base in aqueous solution.

α and β -Glycerylphosphorylcholine

Being aware from our previous work that the chief difficulty in preparing the esters of choline with glycerophosphoric acid lies in the strong affinity of the secondary OH group of glycerophosphoric acid for the N of choline, we tried at first to obtain the esters by starting with glycerophosphoric acid and ethylenimine in anhydrous media, following the model proposed by Christensen (1940) for the synthesis of phosphorylcolamine. We intended to attempt the methylation of the glycerylphosphorylcolamine eventually formed, according to the method of Trier (1912). The ethylenimine was prepared from colamine (obtained according to Knorr, 1897) following the method of Wenker (1935). But our attempt was completely unsuccessful, as we got a white waxy substance with the expected P/N ratio, but which was again a colamine salt of glycerophosphoric acid and not the expected ester. Actually the precipitate with basic lead acetate did not contain N. It was significant in this connexion that, on the contrary, phosphorylcolamine was easily obtained by this method (and, even with better yields, by the direct reaction between phosphoric acid and colamine at high temperature). These

results convinced us that it was very difficult to prevent salt formation between the secondary OH group of glycerophosphoric acid and the amino-

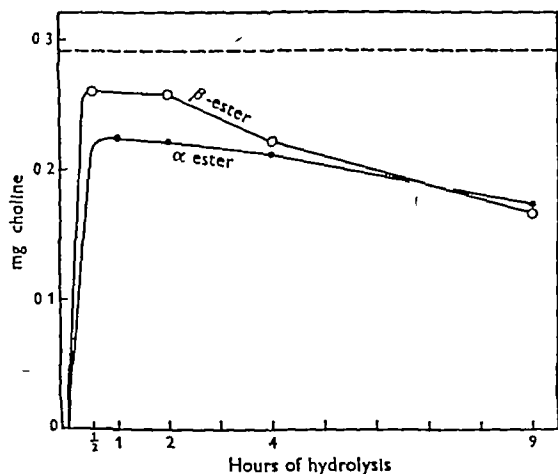


Fig 2 Hydrolysis of synthetic α and β glycerylphosphorylcholine (0.55 mmol.) in 2N HCl. The dotted line is at the theoretical value

group of colamine, or choline, and that apparently this secondary OH group is far more difficult to esterify than the primary one

We then decided to try the picrate of bromocholine and silver glycerophosphate in anhydrous

conditions. We assumed that the presence of the picric acid radical in the bromocholine molecule could prevent the salt linkage with the glycerophosphoric acid, which should be esterified owing to the Ag Br affinity

Preparations We followed the same procedure for both α - and β glycerylphosphorylcholine, using silver α - and β glycerophosphates from the corresponding sodium salts (α isomer 88%, β isomer 100% pure with HIO_4) and bromocholine picrate (m.p. 160°) prepared from bromocholine bromide (m.p. 230°) and picric acid in ethanol

Equimolecular amounts of Ag_2 glycerophosphate and bromocholine picrate were refluxed in absolute ethanol in an oil bath for 60 hr in darkness. The clear ethanolic solution was filtered and evaporated to dryness *in vacuo*. The solid residue was extracted exhaustively with acetone. The dry substance was soluble in water, but no longer soluble in ethanol, it contained Ag. The latter was removed with H_2S from aqueous solution, which was filtered and brought to dryness again *in vacuo* at low temperature. Before complete dryness was reached we found it necessary to neutralize the syrup in order to prevent hydrolysis. The dry residue was a light-yellow sticky syrup, almost insoluble in cold absolute ethanol, but easily soluble in water-ethanol mixtures. For both α and β preparations the yields were extremely low, and as we had to utilize them for biological purposes we did not try to establish other properties than those summarized below

The aqueous solutions were about pH 4, inorganic P and N were absent, P/N ratio = 2.22 (theoretical = 2.21), Florence and reneckate tests were negative, after hydro-

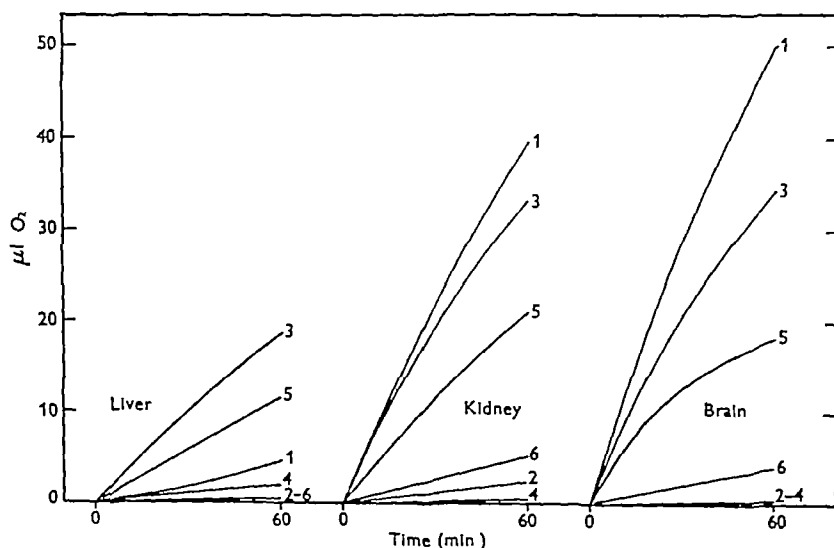


Fig 3 Oxidation of different substrates by guinea pig liver, kidney and brain tissues. The curves show the extra oxygen uptake due to the added substrate. 1 = Na_2 α glycerophosphate, 2 = Na_2 β glycerophosphate, 3 = choline α glycerophosphate, 4 = choline β glycerophosphate, 5 = glyceryl- α phosphorylcholine, 6 = glyceryl- β phosphorylcholine. In every set of experiments, 0.5 ml. of 20% tissue suspensions, homogenized according to Potter & Elvehjem (1936), 1.5 ml. KH_2PO_4 buffer, 0.3 ml. of 0.06M substrate solution (0.0182 mmol.) were used, with physiological saline to 3 ml. Temperature 38°

lysis with 2N HCl at 100° considerable amounts of choline were set free during the first 15–30 min (cf Roman, 1930), these amounts in both α and β glycerylphosphorylcholine were practically consistent with the theoretical figures (see Fig 2), bearing in mind that during the hydrolysis the choline values tend to drop owing to destruction of choline. These results are quite similar to those found by King & Aloisi (1945) and by Schmidt, Herschman & Thannhauser (1945) working with the natural α glycerylphosphorylcholine extracted from beef pancreas. This gave assurance that with this method it is really possible to obtain the choline esters of glycerophosphoric acids. For our biological purposes the method was quite satisfactory for we had sufficient amounts of fairly pure esters to carry on our respirometric measurements, but from the chemical point of view the method is less satisfactory owing to the very low yields. With this method, however, it should be possible to obtain a dicholine ester by working with 1 mol of silver glycerophosphate and 2 mol of bromocholine picrate.

Biological behaviour The oxidations of α - and β -glycerylphosphorylcholine, in comparison with sodium α - and β glycerophosphate, and with choline α - and β glycerophosphate, by living tissues *in vitro* are recorded by us in another paper (Aloisi & Buffa, 1947). We want here only to note (see Fig 3) that in all cases (following Warburg procedure) the α -isomers are better utilized than the β isomers, and that the choline esters and choline salts are oxidized by liver more actively than the sodium glycerophosphates, while other tissues (kidney, brain)

oxidize the simple glycerophosphates better than the esters. In our conditions choline oxidase worked at a very slow rate. Other tests, with bacteria, are in progress.

SUMMARY

1 It is shown that the methods found in the literature for the synthesis of β glycerylphosphorylcholine are likely to give only the choline salts of β glycerophosphoric acid.

2 Choline α and β glycerophosphate (the latter in crystalline form) have been prepared and described.

3 The difficulty in obtaining the choline and colamine esters lay in the fact that ionic reaction preponderated and esterification was prevented.

4 Linkage between choline and glycerophosphoric acid was obtained by treating bromocholine picrate with silver α - and β glycerophosphates under anhydrous conditions.

5 Evidence is given of the formation by this procedure of the choline esters, the α -isomer of which had the same properties as the naturally occurring compound.

6 Preliminary data on the biological behaviour of the esters in living tissues are briefly recorded.

We are greatly indebted to Prof E J King of The Postgraduate Medical School of London, for sending us pure specimens of disodium α and β glycerophosphates.

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Fig 1 Choline β glycerophosphate (1) Method of Arnold from ethanol acetone (2) The same from ethanol pyridine (3) The same from ethanol-ether (4) Method by direct reaction of glycerophosphoric acid with choline, from ethanol ether

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The 267th Meeting of the Society was held in the Department of Biochemistry, University Museum, Oxford, on Saturday, 29 May 1948, at 10 p.m., when the following papers were read

COMMUNICATIONS

Hydrolysis of Organic Compounds by Polarized Radiation By ELIZABETH S. SEMMENS (introduced by R. A. MORTON)

The results of some simple experiments are shown which demonstrate the importance of the orientation factor in physiological reactions and its relation to enzyme action. They comprise

(1) The effect of polarized skylight. The diurnal digestion of starch in the leaf is shown, which takes place under a clear blue evening or morning sky.

(2) Disappearance of starch in the mesophyll in the area exposed behind a Nicol's prism.

(3) Similar hydrolysis is shown to take place in the anthocyanin of a beet leaf, the anthocyanin hydrolyzing to anthocyanidin.

(4) Disappearance of starch in the stomata and its change to a reducing substance. Bursting of the wall of the guard cell by strong polarized sunlight.

(5) The polarizing effect of suspensions of various enzymes on incident radiation is demonstrated, giving the Tyndall effect. Hence the hydrolysis of a suspension of starch grains by a beam of light, polarized by diastase in an outer vessel.

(6) Hydrolysis of starch grains in a suspension of very weak diastase is increased and accelerated by polarized radiation and can be watched under the microscope, but no selective effect is seen if the diastase is active or concentrated. Hence it appears

that both in the case of the enzyme and of the polarized radiation, the physical action is a matter of orientation—probably of the water.

This is further shown by hydrolysis in the absence of enzyme by polarized infra-red radiation, using a weak solution of NaCl (0.05%) or of Ringer's solution, to supply the place of the coenzyme.

(a) A suspension of starch grains in a silica dish, exposed to infra-red radiation, polarized by reflection from a glazed surface, shows transformation of the grains to clusters of crystals of a reducing substance. Further irradiation gives globules which stain with Sudan III and osmic acid.

(b) Films of boiled starch show a lighter patch with iodine solution in the area exposed to polarized radiation.

As it appears to be the water that is activated in all these cases of hydrolysis, no water screen must be used, as thereby the efficient wave lengths would be absorbed.

Further confirmation of these results is given by the use of a double image prism. In both polarized beams, hydrolysis both of film and starch grains takes place, the effect being absent in the crossed beam which gives ordinary light.

Pteroylglutamic Acid Deficiency Induced in Rats by Succinylsulphathiazole and the Inactivity of Anti-Pernicious Anaemia Preparations By E. KODICEK and K. J. CARPENTER (Dunn Nutritional Laboratory, University of Cambridge, and Medical Research Council)

We have been able consistently to reproduce the characteristic pteroylglutamic acid (PGA) deficiency reported to occur in rats fed for 5–6 weeks on a synthetic diet containing 1% succinylsulphathiazole (Daft & Sebrell, 1943). The symptoms were loss of weight, leucopenia and granulocytopenia. If the animals were kept alive with minimal doses of PGA for a further 4–5 weeks a severe anaemia developed, the median erythrocyte count falling from more than 7 to less than 4 million R.B.C./cu mm. of blood, with anisocytosis, increased mean corpuscular diameter and increased mean corpuscular haemoglobin.

A study has been made of the response of deficient animals to single doses of PGA. A response in growth was always accompanied by an increase in

the leucocyte, granulocyte and reticulocyte counts. However, the best dose response relationship was found by plotting the logarithm of the product of the weight increase and the duration of the growth response against the logarithm of the dose given (correlation coefficient 0.88, for 33 oral test doses of 2–100 μ g PGA). PGA given intramuscularly had the same activity.

Other materials have been tested with the following results. Pteroyltriglutamic acid, on an equimolecular basis, had the same activity as PGA. Xanthopterin and 'irradiated' PGA (Carpenter & Kodicek, 1948) were found to be inactive. Marmite had an activity per gram, of the order of 60 μ g of PGA.

Two purified anti-pernicious anaemia liver preparations, supplied by Dr Jacobson, were given to deficient rats *per os* or *im* in nine tests. No response either in growth or in blood picture was obtained at dosages 120 times as great as the lowest effective level of PGA. On the other hand, tested with pernicious anaemia patients, each of the liver extracts, in a single dose of 8 mg, gave a response as great as that given by 100 mg of PGA (Jacobson & Good, 1947). Our results are in keeping with the findings of other workers that liver extracts highly potent for pernicious anaemia are inactive in the PGA-deficient rat (Daft, quoted by Stokstad & Jukes, 1946), chick (O'Dell & Hogan, 1943, Clark, 1945, Stokstad & Jukes, 1946) and mealworm (Fraenkel, 1948).

Jacobson & Good (1947) report that incubation with an enzyme preparation from milk increased the anti-pernicious anaemia activity of PGA. In preliminary trials, using two of their enzyme-treated samples, we found that the response produced in rats was not greater than that obtained with untreated PGA. Kalckar & Klenow (1948) find that incubation with a similar enzyme preparation does not change the activity of PGA as a growth factor for *L. casei* or *S. faecalis* R.

The PGA preparations used in these experiments were 'Folvite' and 'Teropterine' (Pteroylglutamic acid) which were kindly supplied by Dr Jukes of the Lederle Laboratories.

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A Polarographic Study of Pteroylglutamic Acid and Related Compounds By K. J. CARPENTER and E. KODICEK (Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council)

Pteroylglutamic acid (PGA) has been shown to be reducible by catalytic hydrogenation in dilute alkali and the resulting 'dihydro PGA' is readily re-oxidized to the parent compound (O'Dell, Vandenberg, Bloom & Pfiffner, 1947). Xanthopterin has also been found to be reducible to dihydroxanthopterin (Koschura, 1937). We have found that PGA and xanthopterin when electrolyzed at the mercury dropping cathode produce distinct current-voltage curves similar to those reported by Rickes, Trenner, Conn & Keresztesy (1947). The behaviour of these compounds and of 'irradiated' PGA and leucopterin has been studied at different pH.

PGA (Folvite, Lederle Laboratories), 100 µg/ml, in 0.1 N-K₂HPO₄ brought to a pH of 11.3 by adding 0.1 N-NaOH, gave a reduction wave at -1.0 V vs SCE, in absence of air. No reversible oxidation was observed on the charged mercury interface. When tested under the same conditions xanthopterin gave a wave at -1.12 V, and leucopterin at -1.63 V. Solutions of PGA in 0.1 N K₂HPO₄ irradiated by ultraviolet light for 2 hr. and then brought to a pH of 11.3 with 0.1 N NaOH gave four waves at -0.5, -0.82, -1.25 and -1.52 V. Irradiation did not alter the current voltage curve of xanthopterin.

The characteristic waves shifted to the positive

side with increasing acidity. At pH 8.5 both PGA and xanthopterin gave a reduction wave at -0.8 V, whilst 'irradiated' PGA had waves at -0.35, -0.66, -0.79 and -1.34 V. In weakly acid solutions PGA showed two additional reduction waves. At pH 6.1 the original wave had shifted to -0.66 V and the new waves appeared at -1.09 and -1.3 V. At this pH 'irradiated' PGA still showed four waves but now at -0.2, -0.47, -0.6 and -1.12 V.

Stokstad, Fordham & de Gruygen (1947) found that irradiation splits the PGA molecule at the linkage between the pterine moiety and *p*-amino benzoylglutamic acid, with the liberation of 2-amino-4-hydroxy-6-aldehydepteridine (Jukes, 1948, private communication). Neither glutamic acid nor *p*-aminobenzoic acid alone gave any waves at the dropping mercury electrode.

Irradiation of PGA has been shown to destroy its activity as a growth factor for bacteria (Mitchell & Williams, 1944) and for rats (Kodicek & Carpenter, 1948). PGA solutions deteriorate even in ordinary daylight, and we have found that the appearance of these 'extra' reduction waves in the polarogram at pH 11.3 made a convenient test as to whether the activity of a PGA solution has been reduced by exposure to light.

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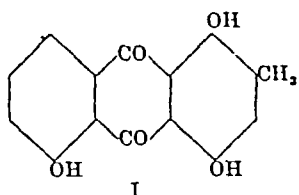
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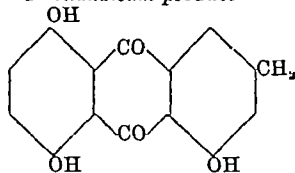
2-Methyl-1,4,5-trihydroxyanthraquinone, a Metabolic Product of *Penicillium islandicum* Sopp By B H HOWARD (introduced by H RAISTRICK) (Division of Biochemistry, London School of Hygiene and Tropical Medicine)

Penicillium islandicum grows well on Czapek Dox 5% glucose solution at 24°. The dried dark red mycelium was extracted with light petroleum followed by chloroform and yielded about 20% of its weight of a complex mixture of fat free colouring matters. These were roughly fractionated by thorough extraction of their ether or chloroform solutions with aqueous Na_2CO_3 . The nature of the Na_2CO_3 soluble pigments is under investigation.

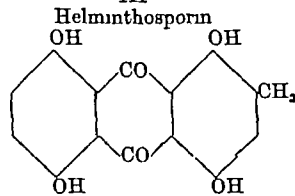
Its constitution was established as follows. It has the molecular formula $\text{C}_{15}\text{H}_{10}\text{O}_5$, contains three OH groups and one (CH_3) C group. It is insoluble in cold Na_2CO_3 but dissolves readily in NaOH. Its colour reactions—intense purple red with red fluorescence with conc H_2SO_4 , deep violet with NaOH, bright yellow orange with green fluorescence in glacial acetic acid—are almost indistinguishable from those given by helminthosporin (structure III). A mixture of it with helminthosporin (m p 225–226°)



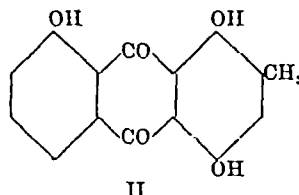
I
P. islandicum product



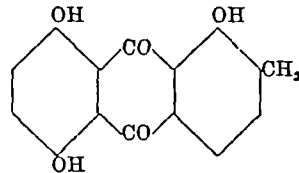
III
 Helminthosporin



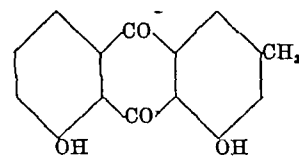
V
 Cynodontin



II



IV



VI
 Chrysophanic acid

The Na_2CO_3 insoluble pigment was extracted with aqueous 0.5N NaOH, recovered by acidification, dried and crystallized from chloroform. 2-Methyl-1,4,5-trihydroxyanthraquinone (structure I, 3% of dried mycelium) was thus obtained as dark red plates, m p 218°, after purification through the triacetate (yellow needles, m p 208°)

melted at 194–195°, a mixture of its triacetate with triacetyl helminthosporin (m p 223–225°) melted at 199–203°

On oxidation with MnO_2 and conc H_2SO_4 it yields cynodontin (structure V, Raistrick, Robinson & Todd, 1933a, Anslow & Raistrick, 1940). Hence it follows that it must have one of the structures I, II,

III or IV Of these III and IV are excluded since III is helminthosporin (Charles, Raistrick, Robinson & Todd, 1933, Raistrick *et al* 1933*b*), and IV has been synthesized and sublimes without melting at 260–270° (Graves & Adams, 1923) It must therefore be either I or II

On reduction with conc HI and red P in glacial acetic acid, followed by oxidation of the resulting

anthranol with CrO_3 in acetic acid, chrysophanic acid (structure VI, Naylor & Gardner, 1931) is formed Of the structures I, II, III and IV the only ones which could give chrysophanic acid (VI) by removal of any one OH group are I and III III is helminthosporin Hence it follows that the *P islandicum* pigment must be I, i.e. 2 methyl 1 4 5 trihydroxyanthraquinone

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The Kinetics of Growth of *Bact lactis aerogenes* in the Presence of Phenol, Alcohols, Ketones and Acetates By S DAGLEY, L. O. FREEMAN and J. O'G. TATTON (introduced by F. C. HAPFOLD)

It was pointed out some years ago (Ferguson, 1939) that the measured physiological action of a drug is determined not only by the 'intrinsic' action, specific for the type of molecule concerned, but is also dependent upon the physical forces governing the distribution of the drug between the cell and the surrounding medium. Thus the toxicities of homologous alcohols increase logarithmically as the series is ascended, not because the molecules of higher members are intrinsically more toxic but, as physico-chemical considerations show, because there is a logarithmic increase with chain length in the number of molecules finding access to sites of action for equimolecular concentrations in the aqueous phase. On the other hand, a comparison of benzyl alcohol with its isomer *p*-cresol indicates that the 'intrinsic' bacteriocidal action of a phenol is several times greater than that of an alcohol.

Two strains of *Bact lactis aerogenes* were used in our experiments. The effect of drugs (including alcohols) acting singly on the first strain was a linear reduction of rate of division in a basal medium, and on the second, the effect was primarily on the lag period. For the former, addition separately of a series of normal alcohols to a culture of cells dividing at reduced rate in the presence of phenol caused an increase in rate to a maximum, followed by a decrease. For the second strain, the addition of ketones and acetates increased growth in the presence of phenol by reducing the lag period, which the latter had caused, down to a minimum value, the lag increasing at higher concentrations of added drug.

Our results may be interpreted on the assumption that the narcotics are adsorbed upon a surface and impede the access of the intrinsically more toxic phenol to its site of action.

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The Adsorption of a Series of Para-substituted Straight-chain Phenols from Solution by Bacterial Suspensions By S DAGLEY, L. O. FREEMAN and W. R. THOMPSON (introduced by F. C. HAPFOLD)

Thick washed suspensions of *Bact lactis aerogenes* were shaken with aqueous solutions of straight chain para-substituted phenols and the uptake of phenol by the cells estimated colorimetrically. The variation in uptake from one member to the next in the series showed a parallel with bacteriostatic activity, which may, therefore, be accounted for by the shift in distribution equilibria as the series is ascended.

Calculation shows that the amount of (unsubstituted) phenol taken up per cell is less than that in the same volume of solution, suggesting that the phenol is adsorbed on the surface and does not penetrate and distribute itself freely throughout the cell. The lag period caused by phenol (0.04–0.06%) in a basal medium supplying nitrogen as ammonium salt can be largely eliminated by the addition of glutamic

acid and methionine (at 0.008%) but not by eight other amino acids investigated at the same concentration. If, under these conditions, the phenol inhibits a rate determining reaction in the synthesis of these acids, it may be inferred that such a reaction does not take place inside the cell.

The addition of ethyl alcohol, acetone or ethyl acetate to suspensions shaken with *p*-*n* propylphenol reduces in each case the uptake of the phenol by the

cells. That this effect is due to surface competition rather than an alteration of the distribution of phenol between lipid and aqueous phases is supported by determinations of the partition coefficient of phenol between olive oil/water in the presence of ethyl alcohol, acetone and ethyl acetate. The first two have little effect on the coefficient, but the presence of the acetate actually *increases* the amount of phenol taken up by the oil.

Identification of the Uronic Acid from Oestriol 'Monoglucuronide' as Glucuronic Acid By J. K. GRANT and G. F. MARRIAN (*Department of Biochemistry, University of Edinburgh*)

Oestriol 'monoglucuronide' was isolated by Cohen & Marrian (1936). Evidence that the substance was a glucuronide was based on carbon and hydrogen determinations, and on a positive Tollen's naphthoresorcinol test.

The method of Lohmar, Dimler, Moore & Link (1942) involving oxidation of the uronic acid to a dicarboxylic acid, which could be identified as the dibenziminazole derivative, appeared to be a method of establishing the identity of the uronic acid with certainty.

Hydrolysis with β glucuronidase was employed in preference to acid hydrolysis, since it was anticipated that the latter might lead to destruction of the uronic acid liberated. Fishman (1939) has shown that β glucuronidase readily liberates oestriol from its 'monoglucuronide', while Levvy (1948) has shown

that its action on menthol glucuronide results in the liberation of glucuronic acid.

Sodium oestriol glucuronide prepared by the method of Cohen, Marrian & Odell (1936), slightly modified, was incubated with ox spleen β glucuronidase (Graham, 1946) at 37° and pH 4.6.

The uronic acid fraction, from the products of hydrolysis, was oxidized with bromine, and the product treated with *o*-phenylenediamine. The dibenziminazole was isolated by a procedure based on those employed by Lohmar *et al.* (1942), and by Levvy (1948). Identity of the iminazole with an authentic specimen of saccharic acid dibenziminazole was proved as shown in Table 1, thus establishing with certainty that the uronic acid from oestriol 'monoglucuronide' is glucuronic acid.

Table 1 *Properties of dibenziminazole and derivatives*

	From Na oestriol glucuronide	From saccharic acid	Mixture
Dibenziminazole*	242–243°	241–243°	242–243°
Dihydrochloride*	265–266° [α] _D ¹⁸ + 49.8° c, 2.057 (water)	266–267° [α] _D ¹⁸ + 49.3° c, 2.042 (water)	265–266°
Dipicrate*	Change of form 142° Melting point 210°	142° 211°	142° 211°

* All melting points are corrected decomposition melting points.

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The Bacterial Oxidation of Phenol to β -Ketoadipic Acid By B. A. KILBY (*Department of Biochemistry, School of Medicine, Leeds, 2*)

A Gram negative vibrio which attacked monohydroxy phenols was isolated by Happold & Key (1932) from sewage containing gas works' liquor. Tyrosine was also attacked without melanin formation, indicating the absence of a tyrosinase system

in this vibrio. It has been shown (Happold, 1930, 1940, Evans, 1947) that catechol and *o*-benzoquinone can be detected as early oxidation products of phenol and formate as a late one, although under favourable conditions complete oxidation to carbon

dioxide and water results. Very little information was available as to the mode of fission of the aromatic ring, except that the culture fluid gave a definite Rothera reaction soon after a colour reaction for catechol became negative. Solubility relationships suggested that the material responsible for the Rothera reaction was an unstable keto acid but not acetoacetic, as it was much less readily extracted by ether from aqueous solution.

It has now been found that culture fluid giving a Rothera reaction can be concentrated *in vacuo* at 37° and pH 7 without decomposition of the keto-acid. Continuous ether extraction of an acidified concentrate caused decomposition of the material responsible for the Rothera reaction, but *lactulonic* acid was isolated from the ethereal extract as its 2,4-dinitrophenylhydrazone, suggesting that the precursor was β ketoadipic acid, and this was eventually isolated by extraction of an acidified concentrate 40 times with ether at room temperature and removal of the ether under reduced pressure. The residue was twice recrystallized from ethyl acetate,

giving β -ketoadipic acid as white needles, m.p. 119–120° (d) unchanged when mixed with an authentic synthetic sample. Both the natural and synthetic acids gave identical and very intense violet Rothera reactions, detectable at 1:100,000 dilution of the acid.

A possible scheme for the oxidation of phenol is

phenol \rightarrow catechol \rightarrow *o*-benzoquinone \rightarrow 1,2,5-trihydroxybenzene \rightarrow 5-hydroxy-*o*-benzoquinone \rightarrow β -ketoadipate \rightarrow succinate + acetate \rightarrow etc.

This pathway is similar to that proposed by Nelson & Dawson (1944) for the tyrosinase oxidation of catechol as far as the 5-hydroxy-*o*-benzoquinone stage, and then instead of this polymerizing to highly coloured products, it is suggested that this compound undergoes a hydrolytic (or phosphorolastic?) fission to β -ketoadipic acid, which may then split into succinate and acetate by analogy with the production of acetate from acetoacetate by *Escherichia coli* observed by Lehninger (1942).

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Studies on Formic Hydrogenlyase in *Escherichia coli* By JUNE LASCELLES (Department of Biochemistry, Oxford)

Washed suspensions of *Escherichia coli*, grown on peptone meat extract glucose broth, showed large losses in formic hydrogenlyase activity when the suspensions were diluted or stored (Lascelles, 1948). These losses were partly restored by addition of extracts of boiled cells, or of small amounts (10^{-3} M) of fermentable sugars or derivatives, such as glucose, fructose, mannose and glucose 1-phosphate. Thus, a fresh suspension with a $Q_{H_2}^{\text{formate}}$ (0–20 min. period) of 150, in the presence of 10^{-3} M glucose, gave a $Q_{H_2}^{\text{formate}}$ of 378 in the same period. After storage of this suspension for 24 hr. at 4°, the $Q_{H_2}^{\text{formate}}$ (0–20 min. period) was 27, and in the presence of 10^{-3} M glucose was 285. Stimulation by glucose was obtained even when formate was added to the suspension after the glucose had been completely fermented.

The glucose appeared to eliminate the initial lag period which was always observed in the course of hydrogen evolution from formate.

Formic hydrogenlyase activity of these suspensions was greatly decreased by incubation in

oxygen for 30 min., or for longer periods, in air. Some of this lost activity was restored by addition of 10^{-3} M glucose.

Preliminary anaerobic incubation of cells with 10^{-3} M glucose resulted in greatly increased formic hydrogenlyase activity of the cells after they had been washed free of glucose. This activity was not further increased by the addition of 10^{-3} M glucose. Incubation under the same conditions with 10^{-2} M formate did not enhance the formic hydrogenlyase activity.

The sensitivity of formic hydrogenlyase to most of the usual inhibitors (Stephenson, 1937) suggests that it is a very complex system. Iron has already been implicated in the formation of this system in *Aerobacter indologenes* (Waring & Werkman, 1944). The involvement of diffusible, heat-stable factors, is indicated by the large losses of activity observed when suspensions were washed in large volumes of phosphate buffer. Activity was largely restored by the addition of extracts of boiled cells. 10^{-3} M glucose alone was ineffective under these conditions.

Activity of the system in borate buffers was very much less than in phosphate buffers of the same pH. Addition of phosphate to borate buffers increased the activity.

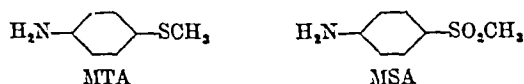
Formic hydrogenlyase activity of suspensions of *E. coli* appeared to be conditioned by the type of peptone used in the growth medium, and by the strain of organism.

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Metabolism of Aryl Sulphides I Conversion of *p*-Methylthioaniline to *p*-Methylsulphonylaniline in the Mouse By F. L. ROSF and A. SPINKS (*Research Laboratories, Imperial Chemical Industries Ltd., Manchester 9*)

During a routine examination of the blood levels attained by a number of aniline derivatives in mice, it was observed that administration of *p*-methylthioaniline (MTA) led to the appearance in the blood of an aromatic amine that, after diazotization, coupled with *N*-β sulphatoethyl *m* toluidine to give



an orange azo dye, whereas the parent compound coupled slowly to give a purple dye when similarly treated. Preliminary tests showed that *p*-methylsulphonylaniline (MSA) behaved like the metabolite, and its acetyl derivative was ultimately isolated from the urine of mice receiving MTA.

Using the analytical methods described in Part II this new metabolic process has been studied quantitatively. Oral administration of 250 mg of MTA/kg to mice gave maximal blood concentrations of 7 mg of MSA and 2 mg of MTA/100 ml. The conversion was extremely rapid, and 20 min after dosing the concentration of MSA was already four times that of MTA. Similar results were

obtained after intraperitoneal administration, showing that the oxidation occurs in mouse tissue and not by bacterial action in the gut. Of an oral dose of 250 mg of MTA/kg about 2% was excreted in the urine as free MTA, 10% as free MSA and 23% as acetylated MSA. No method is available for determining conjugated MTA. The formation of the sulphone in rat and rabbit has been demonstrated by similar quantitative experiments, although no isolation was attempted.

An analogous oxidation of mustard gas has been postulated (Flury & Wieland, 1921) but not confirmed, and has indeed been rejected by some workers on physicochemical grounds (Sugden, quoted by Banks, Bournell, Francis, Hopwood & Wormald, 1946). However, a sulfoxide and a sulphone have been isolated from biological sources (Reichstein, 1936; Reichstein & Goldschmidt, 1936; Ruzicka, Goldberg & Meister, 1940; Pfiffner, 1940) and the existence of sulphide sulphoxide oxidases has been presumed (Medes & Floyd, 1942). We have not studied the sulfoxide corresponding to MTA, and it may be formed as an intermediate metabolite.

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Metabolism of Aryl Sulphides II Simultaneous Determination of *p*-Methylthioaniline and *p*-Methylsulphonylaniline in Biological Material By A. SPINKS (*Research Laboratories, Imperial Chemical Industries Ltd., Manchester 9*)

Two methods of determining MTA and MSA in mixtures have been devised. The first, which could not be applied to biological material, is based on the slow coupling of diazotized MTA with *N*-β sulphatoethyl *m* toluidine (SET). In aqueous solution this

coupling can be completely prevented by a concentration of acid which permits full coupling of diazotized MSA. This allows determination of MSA alone (MSA + MTA) - are determined by reaction with *p*-dimethylaminobenzaldehyde under the conditions

of Morris (1941), when two anils of similar absorption spectra are readily formed. The concentration of MTA is then derived by difference, using a factor to correct for the unequal colour intensities of the anils. In the presence of biological material the coupling of diazotized MTA with SET could not be completely prevented, but the method is the best available for aqueous solutions of the two amines.

The second method utilizes the rapid coupling of both diazonium salts with naphtholsulphonic acids, in the presence of sodium carbonate, to give differently coloured dyes. Satisfactory colour difference and intensity were attained by using *p* carboxy-phenyl-J acid (CPJ). MSA and MTA are determined by fully coupling both compounds with CPJ to give an orange and a bluish red dye respectively.

The optical densities of the mixed dye solution are then read at 450 and 540 $m\mu$ against two standards from pure MSA and pure MTA. At 450 $m\mu$ the optical density of the MSA dye is higher than at 540 $m\mu$, the reverse holds for MTA, so that the concentrations of both can be derived by solving simple simultaneous equations.

Difficulties were encountered when applying the method to biological material, due to the instability of diazotized MTA, the poor recovery of MTA from blood compared to the full recovery of MSA, and a marked effect of blood and urine constituents on the colour of the dye from MTA. These difficulties were solved by using short diazotization times, by adding blood to the standards, and by extracting the compounds from urine with chloroform before determination.

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Purification of Anti-pernicious Anaemia Factor By E. LESTER SMITH and L. F. J. PARKER (Research Division, Glaxo Laboratories Ltd., Greenford, Middlesex)

Ox liver has been concentrated by the methods described by Emery & Parker (1946). Alternatively, proteolyzed liver extract made on a production scale has been reabsorbed chromatographically on to charcoal and eluted with hot 65% ethanol. Material from either source has been further purified by partition chromatography on damp silica, the solvent being *n*-butanol two thirds saturated with water either alone or mixed with phenol. Alternatively *n*- or *iso* propanol containing 10–20% of water gave excellent separations despite the complete miscibility of these solvents with water. The columns usually revealed fast running yellow pigments, and brown pigments held fast near the top, with a pink zone between. Numerous clinical trials showed that this contained substantially all the activity. The red material could be further fractionated from coloured and colourless impurities by repetition of partition chromatography and by fractional precipitation with ammonium sulphate (between one third and two thirds saturation). Some batches of silica adsorb the pigment slightly, permitting additional purification by adsorption chromatography from aqueous solution, or better from one tenth to one half saturated ammonium sulphate, which greatly sharpened the band.

Adsorption chromatography on alumina from aqueous solution (Boots Pure Drug Co., Short & King, 1947) was useful at an early stage to remove

dark pigments. For the partition chromatography, starch could be used instead of silica to avoid the complicating effect of adsorption. On starch columns, red pigments from both proteolyzed and non-proteolyzed liver separated into two pink bands with *R* values averaging 0.5 and 1.2 respectively. Non-proteolyzed liver yielded mainly the slow-moving pigment, the fast moving one, which was also clinically active, is thought to arise from slight autolysis of the liver, because direct papain treatment of the liver yielded mainly this pigment. Repeated chromatography effected little or no further concentration of the colour beyond the stage where an optimal clinical response (Emery & Hurran, 1945) is given by about 0.3 mg. Some chemical and physical properties of such material have recently been described (Lester Smith, 1948). Following treatment with trypsin, however, chromatography on silica or charcoal gave further dramatic concentration. The substance finally crystallized from aqueous acetone as red needles. The crystalline product had 37 times the colour intensity of a batch active at 0.3 mg, giving a calculated dose of 8 μ g.

It appears probable that our substance is the same as that recently described by Rickes, Brink, Koniuszy, Wood & Folkers (1948) with the suggestion that it be given the name vitamin B₁₂.

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Sulphur Compounds of *Allium* Detection of *n*-Propylthiol in the Onion The Fission and Methylation of Diallyl Disulphide in Cultures of *Scopulariopsis brevicaulis* By F CHALLENGER and D GREENWOOD (Department of Organic Chemistry, The University, Leeds)

In the course of a study of the volatile ingredients of onions a stream of sterile air was passed over freshly peeled and chopped onions and then through (a) aniline, (b) 2N HCl, (c) 4% aqueous mercuric cyanide and (d) 3% aqueous mercuric chloride to remove isothiocyanates, aniline vapour, thiols and sulphides and disulphides respectively.

In three separate experiments with the onions a solid formed in the mercuric cyanide after 6 hr and increased no further after 2-3 days. It had m.p. 63-65°, and after one crystallization from alcohol, m.p. 66-67°. In admixture with authentic mercury di-*n*-propylthiol, $\text{Hg}(\text{SCH}_2\text{CH}_2\text{CH}_3)_2$ of m.p. 67°, the m.p. was 67-68°. The other two experiments gave similar results (Found C, 20.8, H, 4.05. Calc for $(\text{C}_3\text{H}_7\text{S})_2\text{Hg}$ C, 20.5, H, 4.0. The diallyl derivative $(\text{C}_3\text{H}_5\text{S})_2\text{Hg}$ requires C, 20.7, H, 3.0%). Mercury diallylthiol, moreover, melts at 74°, and the mixed melting point with the recrystallized deposit from the mercuric cyanide was 60-61°. The volatile product is therefore *n*-propylthiol. Conversion of the mercury derivative to the corresponding lead and silver compounds and comparison (melting point and/or mixed melting point) with authentic specimens confirmed this conclusion.

The solid which formed in the mercuric chloride during the passage of air over the onions was much less than that in the cyanide, indicating the absence of appreciable quantities of readily volatile sulphides or disulphides or, alternatively, that any such compounds present react only slowly with mercuric chloride. (The boiling point of *n*-propylthiol, *n*-propyl sulphide, di-*n*-propyl disulphide, allyl sulphide and diallyl disulphide are 67°, 141.5-

142.5°, 192.5°, 138-139° and 79° at 16 mm respectively.)

Semmler (1892) found no allyl sulphide in oil of onions, but concluded that the main constituent was probably propyl allyl disulphide. Challenger & Rawlings (1937) showed that, in cultures of *Scopulariopsis brevicaulis*, di-*n*-propyl disulphide yields *n*-propylthiol and *n*-propyl methyl sulphide. So far as the authors are aware no other biological formation or occurrence of *n*-propylthiol has been recorded, nor has a thiol previously been detected in the onion, though Semmler (1887) detected traces of a thiol in the oil of *Allium ursinum*.

Semmler (1892) states that diallyl disulphide occurs in oil of garlic. As a study of the behaviour of a plant constituent under other biological conditions might furnish useful analogies, diallyl disulphide in sterile aqueous suspension was added to bread cultures of *Scopulariopsis brevicaulis* under the conditions employed by one of us with Rawlings (1937) and Blackburn (1938). Volatile products were aspirated through (a) aqueous 4% mercuric cyanide and (b) aqueous platonic chloride. Mercury diallylthiol $(\text{C}_3\text{H}_5\text{S})_2\text{Hg}$, m.p. and mixed m.p. 74°, with an authentic specimen of the same melting point separated in the cyanide. The identity was checked by conversion to the lead and silver derivatives and comparison as before. The platonic chloride compound of methyl allyl sulphide, $(\text{CH}_3\text{SC}_3\text{H}_5)_2$, PtCl₄, m.p. (after crystallization) and mixed m.p. 150° with an authentic specimen of the same melting point, was obtained from the platonic chloride absorption tube (Found Pt, 38.0. $\text{C}_8\text{H}_{16}\text{S}_2\text{Cl}_4\text{Pt}$ requires Pt, 38.0%). A second experiment confirmed these results.

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The Faecal Lipoids of Rats Fed on a High Cholesterol Diet By R. P. COOK, N. POLGAR and R. O. THOMPSON (Department of Biochemistry, University College, Dundee, and Dyson Perrins Laboratory, Oxford)

Rats placed in metabolism cages were fed on synthetic diets containing various fats alone and with the addition of 2% cholesterol. The faecal lipoids were separated into unsaponifiable matter (U.M.) and acids. The results obtained are shown in Table 1.

There is an increase in the excretion of acids on the cholesterol containing diets (Cook, 1938). This increase is approximately equal in amount to the cholesterol unaccounted for if a balance of the sterol is made.

For separating the faecal acids a scheme was employed which was developed in connexion with another investigation (Polgar, 1948) and depends

was isolated by converting its hydrogen succinate into the acetol ester and then into the semi-carbazone

Table 1

Dietary fat	Added chole sterol	Faecal lipoids (g)	U M (g)	Acids (g)
Butter fat	0	5.9	1.9	3.8
	22.7	30.9	20.4	7.7
Olive oil	0	12.6	4.0	6.8
	36.5	44.0	29.1	11.3
Cooking fat	0	6.7	3.0	3.3
	19.1	24.6	16.6	6.8

Table 2 Sterol (as U M) balance with animals on butter fat diet

	U M in livers (g)	U M in faeces (g)	Total A	Dietary chole sterol B (g)	B-A	Faecal acids
Cholesterol fed	0.40	20.4	20.8	22.7	—	7.7
Controls	0.08	1.9	2.0	—	—	3.8
Difference	0.32	18.5	18.8	22.7	3.9	3.9

upon the crystallization of derivatives obtainable by converting the acids into acetol esters, $R \cdot CO_2CH_2CO \cdot CH_3$, and treating the latter with a reagent for ketones. From the unsaponifiable material the sterols were separated by crystallization of the 3:5 dinitrobenzoates, one of the components

The faecal acids contain, in addition to the common fatty acids, a dextrorotatory acid of high molecular weight. The latter is found only in the faeces of the cholesterol fed animals, irrespective of the dietary fat fed, thus indicating its probable metabolic relationship to cholesterol.

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A Study of the Peptides of Cystine in Partial Acid Hydrolysates of Wool By R. CONSDEN and A. H. GORDON (Wool Industries Research Association, Headingley, Leeds 6)

Because of easy oxidation to sulphonic acids, cystine and peptides of cystine are readily adaptable for isolation by anionic exchange resins and fractionation by ionophoresis. Oxidation was therefore employed in one of the stages of this investigation, which was carried out according to the following general scheme. A partial hydrolyzate of wool, prepared by treatment with 10N HCl for 10 days at 37°, was fractionated on a chromatogram of Amberlite IR 4 (Consdén, Gordon & Martin, 1948) to remove acidic amino acids and peptides. The solution was then oxidized with Br₂ water and retreated with Amberlite to remove neutral and basic constituents. The mixture of cysteic acid and peptides of cysteic acid thus obtained was next subjected to ionophoresis (Consdén *et al.* 1946), which gave eight fractions (A-H) moving towards the anode. Each fraction was then worked up for analysis on paper chromatograms as described by Consdén *et al.* (1947).

The fastest moving fractions, A and B, contained small amounts of di- or tripeptides of cysteic acid with one or two residues of a dicarboxylic amino acid. Fraction C consisted of free cysteic acid. Fraction D contained dipeptides, of which cysteic-glycine (β sulphoalanylglycine) and cysteic-alanine were identified. Fraction E contained glycylcysteic acid, serylcysteic acid, alanylcysteic acid, threonylcysteic acid, cysteic-valine, cysteic-leucine and probably cysteic-threonine in relatively small amount. Results for fractions F, G and H were more difficult to interpret owing to the large number of amino acids obtained after hydrolysis of some of the peptide spots extracted from the paper chromatograms. However, leucylcysteic acid and phenylalanylcysteic acid were identified in fraction F, which also appeared to contain tripeptides, of which one, consisting of cysteic acid linked with proline and valine in unidentified residue order, was probably present. Fraction G probably consisted of

tri and higher polypeptides of cysteic acid and was rich in peptides containing proline and the higher monoamino monocarboxylic acids. The slowest moving fraction H probably contained polypeptides consisting of many amino acid residues, since hydrolysis of some of the spots extracted from paper chromatograms gave lysine and arginine in addition

to neutral and acidic amino acids. This fraction contained dibromotyrosine (probably free) and was also rich in peptides containing proline residues.

Dipeptides of cysteic acid are being synthesized in order to compare their ionophoretic and chromatographic behaviour with those found in the oxidized wool hydrolysate.

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The Oxidation of L-Ornithine in the Presence of Belladonna Polyphenolase By W. O. JAMES and H. BEEVERS (*Oxford Medicinal Plants Scheme*)

A polyphenolase that is insoluble after precipitation with acetone has been extracted from the leaves and roots of *Atropa belladonna*. It may be washed until free from polyphenols and secondary substrates such as amino acids without appreciable loss of activity. Suspended in buffers it rapidly oxidizes catechol with the uptake of two atoms of oxygen for each molecule of catechol oxidized.

Following Nelson & Dawson (1944), it is presumed that the oxidation product is *p*-hydroxy *o*-quinone. On standing, brown pigments are formed that are incapable of reduction to catechol. The addition of an α amino acid at any time before the catechol has been fully converted to pigments gives rise to a strong red coloration. With a molecular ratio amino acid/catechol = 1, the colour reaches almost maximum intensity and there is no oxidation of amino acid. The use of substituted catechols shows that no colour compound is possible if the two para positions are blocked (e.g. as in aesculetin) and the pigment is regarded as a *p*-imino *o*-quinone. L-Ornithine enters into this reaction as well as a long list of other amino acids investigated. Secondary amines form tertiary nitrogen compounds of similar structure and purple colour. As was found earlier with glycine, the first molecule of L-ornithine is not oxidized but remains condensed in the colour com-

pound which has about the same degree of stability as the glycine colour compound. Further condensation to brown pigments is very much slower than in the absence of an amino acid.

Any excess of glycine over the 1/1 ratio with catechol leads to at least partial oxidation of the excess amino acid. Other amino acids tried are oxidized much less actively, or not at all. L-Ornithine is exceptional and is oxidized at about one third the rate of glycine, and approximately 1 mol of ammonia is released for each additional atom of oxygen consumed. The oxidation product has been identified as α -keto δ -amino valerianic acid. L-Lysine is also oxidized.

Acetylation of the α amino group of L-ornithine, omission of the δ amino group as in α -aminovalerianic acid or substitution, as in L-arginine or L-citrulline, greatly reduces the oxidation. In spite of the high degree of specificity, the oxidation is non-enzymatic and is catalyzed by the colour compound in pure solution. It appears to result from the additional molecular instability caused by the introduction of a δ amino group adjacent to the active groups common to all amino acids.

L-Proline behaves as a secondary amine in this system. It forms a strong purple colour but is not oxidized.

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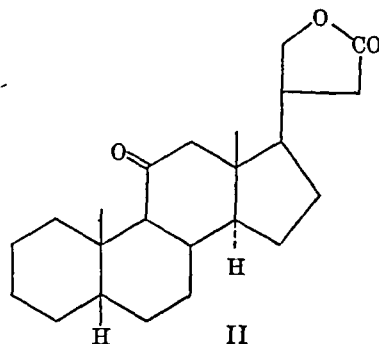
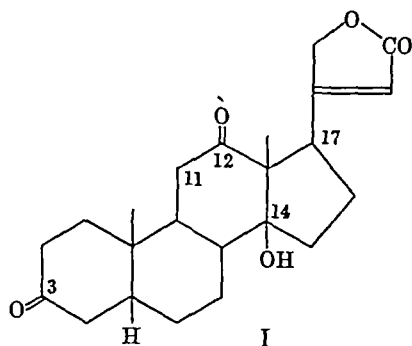
Observations on the Structure of Sarmentogenin By W. KLYNE (*Biochemistry Department, Postgraduate Medical School of London*)

Sarmentogenin and digoxigenin are isomeric diglucosides, $C_{42}H_{64}O_{16}$, which on oxidation with CrO_3 give isomeric diketones sarmentogenone and digoxigenone, each possessing one inert keto group. Digoxigenone is known (Steiger & Reichstein, 1938,

Mason & Hoehn, 1939) to be I, the work of Tschesche & Bohle (1936) suggested that sarmentogenone might be the corresponding 3,11-diketone. Katz & Reichstein (1944) compared the molecular rotations of the two genones with those of known 11- and

12-keto compounds The difference between the molecular rotations of the two genones ($+413^\circ$) is much greater than the differences for pairs of known com-

parisons may, however, be made using the rotation differences between deoxotetrahydroanhydrosarmentogenone (II?) and its derivatives (which are



pounds (C_{12} ketone minus C_{11} ketone = $+152^\circ$ to $+212^\circ$) and the structure of sarmentogenin was therefore considered uncertain

14 *n*, and carry a butanolide ring at C_{17} , Tschesche & Bohle, 1936), and those for transformations at C_{11} in bile acid esters of known structure (see table)

Structural differences	Molecular rotation differences	
	Bile acid esters (transformations at C_{11})	Deoxotetrahydro- anhydrosarmento- genone and derivatives
$C=O$ minus CH_2	$+58^\circ$ to $+90^\circ$	$+41^\circ$
$C=O$ minus $CHOH$	-37° to $+32^\circ$	-19°
$CHOH$ minus CH_2	$+61^\circ$ to $+115^\circ$	$+60^\circ$

This comparison appears to be invalid, since (a) the genones are 14 *iso* compounds while the 'known' compounds are 14-*n*, and (b) the butanolide ring of the genones at C_{17} might exert 'vicinal action' at C_{11} and C_{12} (cf Barton & Cox, 1947) Valid com-

These comparisons, when taken in conjunction with the inert character of one keto group of sarmentogenone, support the hypothesis that sarmentogenin and its derivatives carry an oxygen atom at C_{11} .

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The Administration, Storage and Metabolism of Vitamin A By J GLOVER and R A MORTON (Department of Biochemistry, University of Liverpool)

The blood level of the functional vitamin can be raised by administering large doses of vitamin A concentrate (Glover, Goodwin & Morton, 1947) but the method is wasteful, since the liver vitamin reserves built up in that way are lost fairly rapidly (Davies & Moore, 1935) When carotene is administered over a period the resulting vitamin A store persists much longer (Dann & Moore, 1931) In the first experiment a large amount of the vitamin is absorbed in a short space of time, whereas in the

second the vitamin, formed from the provitamin in the intestine (Glover *et al* 1948), is absorbed in small amounts slowly over a longer period

Now, using a fluorescence microscopy technique, Popper & Greenberg (1941) have observed two distinct types of fluorescence attributable to vitamin in liver If, as suggested by Glover *et al* (1947), the diffuse type of fluorescence spread over the cytoplasm of the true storage cells is due to a diffusible alcohol complex, then his results mean that only those cells

contain the free vitamin in equilibrium with ester whereas the Kupffer cells contain merely the ester form

Recent evidence of Glover *et al* (1947) shows that as the concentration of the vitamin A dose administered to the rat is increased, the ratio of ester/alcohol form in liver tends to rise from 80/20 (undosed rats) to c 95/5 (rats at the highest dose level) This apparent change in equilibrium of the reaction $\text{vitamin A ester} \rightleftharpoons \text{vitamin A alcohol} + \text{fatty acid}$ can be satisfactorily explained on the assumption that there are two distinct sites of storage in the liver, one of which does not contain lipase The Kupffer cells could form this lipase free store, and, in fact, by a histological method, Gomori (1946) has shown the absence of lipase from these cells

The above observations can be reconciled as follows As the dosage of vitamin A rises, i.e. as the rate of entry of vitamin into the blood stream increases, the amount finding its way into the *K* cells increases Hence, it appears that during the absorption of vitamin A (or fat) from the blood into the liver two processes are operating simultaneously

(i) hydrolysis of incoming ester by serum esterase or lipase which releases free vitamin A to form a complex capable of diffusion into the liver storage cells, and (ii) phagocytic action of *K* cells on the chylomicrons trapped in the liver sinusoids as the blood flows through them Vitamin A stored in the *K* cells does not contribute to the level of functional vitamin in the blood Indeed, the evidence of depletion experiments suggests that it is destroyed there

This explanation has wide implications

(i) There is an optimum rate of ingestion of vitamin A such that the bulk of absorbed material should find its way into the true storage cells where it will form a more lasting reserve and can supply the functional vitamin to the blood

(ii) Carotene, in spite of variable absorption, may be more effective than vitamin A concentrates, inasmuch as newly formed vitamin from the intestine may trickle into the blood stream at a rate matching the capacity for its entry into true liver cells

(iii) It is better to administer vitamin A in a series of small doses rather than in one large single dose

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The Enzymic Hydrolysis of Triacetin (Glyceryl Triacetate) By PAMELA HOLTON (introduced by H BLASCHKO) (Department of Pharmacology, Oxford)

Three different preparations of cholinesterases have been found to hydrolyze triacetin cobra venom (Bovet & Bovet, 1943, Bovet Nitti, 1947), the caudate nucleus of the dog's brain (Bodansky, 1946) and horse serum

The rate of hydrolysis of benzoyl choline and triacetin by horse serum were additive when both substrates were added simultaneously Nu 683 (Gunter & Hawkins, 1946) inhibited the cholinesterase but had no effect on triacetin hydrolysis by horse serum These results suggest that triacetin esterase is not identical with pseudocholinesterase, this was confirmed by the inability of a highly

purified preparation of pseudo cholinesterase to hydrolyze triacetin

Competition for both cobra venom and dog brain occurs when triacetin and acetylcholine are added together to the enzyme preparations In both preparations selective inhibitors of cholinesterase also inhibit triacetin hydrolysis All these experiments are compatible with the interpretation that when triacetin is hydrolyzed by the tissue of the caudate nucleus or by cobra venom, the hydrolysis is effected by the cholinesterase present

The purified preparation of pseudocholinesterase was kindly prepared by Dr J W Legge

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The Effects of a Relative Deficiency of Lysine and Tryptophan in the Diet of an Insect, *Tribolium confusum* By G FRAENKEL (*Department of Zoology and Applied Entomology, Imperial College, London, S W 7*)

It has recently been shown (Krehl, Sarma & Elvehjem, 1946) in work with rats that diets containing a low, but still adequate amount of casein can be made inefficient by the addition of a protein of low tryptophan content, and that such induced deficiencies are remedied by the addition of tryptophan or nicotinic acid. We have obtained results of a similar kind in work with the flour beetle, *Tribolium confusum*. With zein as the sole source of protein no growth ensues. This deficiency is rectified only by the addition of both lysine and tryptophan. A diet with 20 % casein becomes inferior by the addition of 20 % zein. This casein-zein diet is somewhat improved by the addition of lysine but not of tryptophan, while with the addition of both amino acids the diet becomes superior to the casein diet.

A similar relationship is shown by gliadin, by itself a very inferior source of protein, which is also

greatly improved by the addition of lysine, but not by that of tryptophan. A 20 % casein-20 % gliadin diet again is much inferior to a 20 % casein diet and is improved by the addition of lysine, but not of tryptophan.

These phenomena of induced tryptophan and lysine deficiencies occur in the presence of relatively large amounts of nicotinic acid and there is no indication of an interchangeable effect of tryptophan and nicotinic acid. The effects recorded in these tests with *Tribolium* should therefore be interpreted rather in terms of impaired balance of essential amino acids in the diet than in terms of differential utilization of an amino acid in respect of which a diet is marginal, which, according to Krehl, Huerga & Elvehjem (1946), is the basis for the explanation of the tryptophan-nicotinic acid relationship in the rat.

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The Rate of Enzymic Hydrolysis of Phosphoric Esters by Prostatic Phosphatase By G. E. DELORY

Studies of the rate of enzymic hydrolysis of a series of phosphoric esters by the alkaline phosphatase of dog faeces (King & Delory, 1939; Delory & King, 1943) showed that the rate of hydrolysis increases with increasing acidity (decreasing pK) of the substrate and that the enzyme is optimally active at a more alkaline pH.

The acid phosphatase of human seminal fluid has now been studied in a similar manner. The following esters in order of increasing pK were employed: *p*-bromophenyl, 2,4-dibromophenyl,

o-bromophenyl, *p*-nitrophenyl, phenyl, *o*-cresyl, 2,4,6-tribromophenyl, β -glycero, α -glycero and ethyl phosphate. In general the rate of hydrolysis increases with increasing acidity of the ester and the enzyme is optimally active at a more acid pH, although the figures obtained depend on the particular enzyme preparation used.

The effect of varying the substrate concentration was also investigated. Although the rate of hydrolysis is dependent upon the substrate concentration, the Michaelis-Menten equation is not closely followed.

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The Specificity of the Human Erythrocyte Cholinesterase By D. H. ADAMS and V. P. WHITTAKER (*Department of Biochemistry, Oxford*)

Following the work of Alles & Hawes (1940), Mendel & Rudney (1943) suggested the division of cholinesterases into 'true' and 'pseudo' types. True cholinesterases (from brain and erythrocytes) were stated to be active only against acetylcholine and closely related substances, while the pseudo enzymes

(e.g. from horse serum) are capable of hydrolyzing both choline and non-choline esters. Nachmansohn & Rothenberg (1945) also concluded that cholinesterases may be divided into 'specific' and 'non-specific' types. However, as far as non-choline esters are concerned, these conclusions appear to have been

based almost exclusively on the failure of brain and erythrocyte cholinesterases to hydrolyze tributyrin and methyl butyrate

Starting from the observation of Bodansky (1946), that brain, and purified (Mendel & Rudney, 1943) erythrocyte preparations, nevertheless rapidly hydrolyzed triacetin, we have examined the activity of similarly purified human erythrocyte preparations against a number of simple esters, and found that many are hydrolyzed, although in general not at very high rates. Summation experiments have shown that in all probability the preparations contained only a single enzyme

The results suggest that the specificity of the erythrocyte cholinesterase is influenced by at least two factors

(1) The acyl group. Acetates are hydrolyzed more rapidly than the corresponding propionates or formates, while esters containing butyryl radicals are hardly attacked, if at all

(2) The alcohol group. Considering acetates, lengthening the alcohol chain up to four atoms (*n* butyl) increases the rate of splitting, while a further increase in chain length (*n* amyl, *n* hexyl) leads to a reduction. Extra C atoms accommodated as

branches which are some distance away from the ester link (isoamyl, 2 ethylbutyl) considerably increase the rate of hydrolysis. Branching close to the ester link appears to reduce the rate. Significantly acetylcholine contains a 'spine' C—C—N—C with branches. Mono and triacetin appear to be special cases

Published data upon the specificity range of the human plasma cholinesterase suggests that in contrast to the erythrocyte enzyme, the plasma enzyme accommodates preferentially the larger acyl groups such as butyryl and propionyl. Acetyl and benzoyl derivatives are attacked, but not so readily. The effects of chain branching appear to differ in the two systems, since it is stated (Alles & Hawes, 1940) that while human plasma hydrolyzes acetyl α methylcholine, but not acetyl- β methylcholine, human erythrocytes are capable of hydrolyzing both. A tentative hypothesis is advanced to explain such differences in substrate specificity

It would thus appear inappropriate to continue to refer to the human erythrocyte enzyme as a 'specific' cholinesterase. It is hoped to continue this work by the examination of brain preparations

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The Effect of Sulphydryl-combining Reagents on the Activity of the Succinic Oxidase System.

By E. C. SLATER (*Molteno Institute, University of Cambridge*)

The action of sulphydryl combining reagents on the enzyme system catalyzing the oxidation of succinate has been widely studied. It is generally believed that these reagents react with an essential —SH group or groups on the succinic dehydrogenase protein. Hopkins & Morgan (1938) and Barron & Singer (1945) found that the inhibition could be readily reversed by simple thiols, other authors (Potter & Dubois, 1943; Ames & Elvehjem, 1944) have, however, failed to obtain this reactivation.

In order to obtain the reactivation it is necessary to work under anaerobic conditions, since, in the presence of air, thiols inactivate the succinic oxidase system by destroying a factor linking the succinic dehydrogenase with the cytochrome oxidase (Slater, 1948).

p-Aminophenylarsenoxide affects only the succinic dehydrogenase portion of the system, and the inhibition of either the complete system or of the

succinic dehydrogenase portion is readily reversed by small concentrations of 2,3-dimercaptopropanol (BAL) and partially by higher concentrations of glutathione. Oxidizing agents (oxidized glutathione, *o*-iodosobenzoate, cupric salts), on the other hand, affected some other portion of the system in addition to the succinic dehydrogenase, and the inhibition of the complete succinic oxidase system was less readily reversed than that of the succinic dehydrogenase. It is possible that this other portion is the factor mentioned above. *p*-Chloromercuribenzoate also inhibited the complete succinic oxidase system more than the succinic dehydrogenase. It also had some effect on the cytochrome oxidase. It is believed that this mercurial, in addition to combining with the —SH group of succinic dehydrogenase, has a general effect on proteins and affects the particles of the enzyme preparation (heart muscle preparation of Keilin & Hartree, 1947) in such a way as to affect the accessibility of the succinic dehydrogenase to the

cytochrome oxidase Inhibition by the mercurial was readily reversed by cyanide, glutathione and denatured globin but not by BAL

The mercurial differed from the other sulphhydryl reagents used in two other respects, viz (1) substances present in the heart muscle preparation which completely protected the succinic oxidase system from small amounts of the arsenical or

oxidizing agents gave no protection from the mercurial and (2) the rate of inactivation was much greater in the case of the mercurial All these differences are probably related to the fact that arsenicals and oxidizing agents react with two —SH groups (either on the same succinic dehydrogenase molecule or on different molecules), while the mercurial reacts with only one —SH group

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Further Observations on the Effect of Cetyl Trimethyl Ammonium Bromide on the Bacterial Decarboxylation of Glutamate and Glutamine By D E HUGHES (*Medical Research Council Unit for Cell Metabolism, Department of Biochemistry, The University, Sheffield 10*)

It has previously been reported (Krebs, 1948) that the decarboxylation of glutamic acid and of glutamine by washed suspensions of *Clostridium welchii* is accelerated by cetyl trimethyl ammonium bromide ('cetavlon') It was thought that this acceleration might be due to a change in the permeability of the cell wall allowing a more rapid entry of the substrate into the cell, and if this were the case, the detergent should have no effect in cell-free extracts To test this, extracts were prepared by grinding the centrifuged cells with powdered pyrex glass (McIlwain, Roper & Hughes, 1948) and adding 6 vol of 0.04M-acetate buffer, pH 4.6 The supernatant obtained on centrifuging contained from 50 to 60% of the decarboxylase activity and from 40 to 50% of the glutaminase activity of the washed cells and showed no appreciable loss of activity within 9 days when stored at 0° The effect of cetavlon in these extracts was found to be of the same order as in the intact cells as shown in the following table

0.011M in the absence of cetavlon and 0.0045M in the presence of cetavlon (pH 4.1) It would thus appear that the affinity of the enzyme for the substrate is increased by the detergent

A comparison of the rates of decarboxylation at different concentrations of cetavlon showed that the effect was maximal at a concentration at which the detergent changed methyl orange from an orange to a yellow colour This colour change which is not due to a change in pH is generally regarded as a result of micelle formation (Hartley, 1934, Corrin & Harkins, 1947) Effects similar to those of cetavlon were observed with homologous alkyl trimethylamine bromides (C_{10} , C_{12} , C_{14} , C_{16}) and with other cationic detergents, and in every case a maximum effect on the decarboxylase occurred when the concentration was sufficient to change the colour of methyl orange The observations suggest that the effect of cetavlon is connected with the formation of micelles of the detergents

Effect of cetavlon on the rate of decarboxylation by Clostridium welchii, 40°, pH 4.1

Substance added	Q_{CO_2}	
	Intact cells	Extract
Glutamate 0.0045M	270	224
Glutamate 0.0045M, cetavlon 0.0025M	535	405
Glutamine 0.0045M	114	0
Glutamine 0.0045M, cetavlon 0.0025M	270	290

It follows that the effect of cetavlon cannot be explained by changes in permeability Determination of the rate of NH_3 formation from glutamine showed that both glutaminase and glutamate decarboxylase were accelerated by cetavlon

The apparent Michaelis constant of the extract for the decarboxylation of glutamate was found to be

The glutamic decarboxylase and glutaminase from *Proteus morganii* and *Escherichia coli* behaved similarly, but the decarboxylase from carrot and squash (Schales, Mims & Schales, 1946) was not affected by cetavlon, neither were the decarboxylases for arginine (*Esch. coli*), lysine (*Esch. coli*), tyrosine (*Streptococcus faecalis*) and ornithine (*Clostridium septicum*)

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Methods for Estimating Total Serum Protein By B B LLOYD, H M SINCLAIR and M C K TWEEDIE (Wellcome Laboratory of Human Nutrition, Oxford)

In a previous communication we concluded that the published equations for density-protein conversion were inadequate for normal human serum, and the CuSO_4 method of estimating density had serious defects (Lloyd, Cheek, Sinclair & Webster, 1945). Two months later we carried out extensive examinations in the Netherlands during the famine and, because we were surprised to find that persons with famine oedema had values for total serum protein within the statistically normal range, we compared the density method we were using (Jacobsen & Linderstrom Lang, 1940) with three other methods

gravimetric, micro Kjeldahl and biuret. The gravimetric estimations were made by Mr v d Kamer and Mr Bos with the method of Robinson & Hogden (1941). After using with good agreement three modifications of the micro Kjeldahl method, we adopted that of Chubnall with a digestion period of 2 hr.

The results permit of a comparison of the four methods. An equation can be derived relating relative density to protein content estimated gravimetrically, and compared with the various published equations.

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Estimations of Serum Protein in Famine Oedema By B B LLOYD, H M SINCLAIR and M C K TWEEDIE (Wellcome Laboratory of Human Nutrition, Oxford)

The previous communication related to estimations of serum protein by four methods in persons without clinically demonstrable famine oedema, the present results show that two of the methods (micro-Kjeldahl and densitometric) give anomalous results when samples from persons with famine oedema are used.

Govaerts & Grégoire (1941) found that the quality of the serum protein in persons with famine oedema was altered, since the observed colloidal osmotic pressure was lower than that calculated from the amounts of albumin and globulin. Florkin & Duchâteau (1947) found that in certain persons with famine oedema the arginine/lysine ratio for total plasma protein was decreased.

In our estimations in Holland we found that when the gravimetric estimation of total serum or plasma protein was taken as the reference, there was no difference between values for persons with or without famine oedema when the biuret method was used,

but the micro Kjeldahl and densitometric methods showed that the protein from persons with oedema contained less nitrogen and contributed more to serum density than the protein of persons without oedema. Estimations of protein in serum or plasma or oedema fluid from persons with famine oedema by the gravimetric and densitometric methods gave a correlation coefficient of 0.991 ($n=22$), the equation relating the two sets of values is as follows:

$$\text{gravimetric protein in g/100 ml of fluid} = 354(d_{20}^{20} - 1.0073),$$

and agrees closely with that found by Weech, Reeves & Goettsch (1936) for serum, plasma and transudate of dogs, many of whom were hypoproteinaemic.

It is clear that there is a qualitative change in the serum protein in famine oedema, although, as we have previously shown (see Sinclair, 1947), the oedema is not caused by a lowered concentration of serum protein.

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Obituary Notice

JOHN MASSON GULLAND, 1898-1947

British science sustained a grievous loss by the tragic death of Prof J M Gulland in a railway accident at Goswick, near Berwick on Tweed, on 26 October 1947. He was a biochemist whose work was founded on a solid basis of achievement in the field of pure organic chemistry, and he will be remembered above all for his work on nucleic acids. His contributions to the chemistry and biochemistry of these important cell constituents have greatly enriched our knowledge of them and will rank very highly among the pioneer investigations in this field. Gulland was a man of vigour and vivacious charm, of whom Dr Jesse P. Greenstein has written (*Nature, Lond.*, 1948, 161, 87) 'Gulland was one of a group of distinguished foreign guests at the Cold Spring Harbor Symposium held in June 1947. He was easily the dominant figure at that conference, and the charm and ease of his manner, his gentle critical spirit, together with the scholarliness and incisiveness of his thinking, evoked general admiration and affection.' This spontaneous tribute from across the Atlantic describes Gulland in terms which recall very vividly the man whom his friends admired and respected.

John Masson Gulland was born in Edinburgh in 1898 and was the only son of the late Prof G. Lovell Gulland, Professor of Medicine in the University of Edinburgh. He was devoted to his native city and had an intense love of Scotland. He had a wide and detailed knowledge of the Scottish Highlands, their local customs, history and geology, and he made some delightful water colour sketches during his many holidays there. He was also a keen fisherman in Scottish rivers. Gulland was educated at Edinburgh Academy and the University of Edinburgh, where he graduated in 1921, after serving as a second lieutenant in the First World War. Having been awarded a Carnegie Scholarship, he went to work at St Andrews under Prof. (later Sir) Robert Robinson, whom he rejoined in Manchester, again later in Oxford. Gulland was in the Dyson Perrins laboratory when Robinson was appointed to the chair of chemistry. Gulland became a University demonstrator in chemistry at Oxford in 1924, and was appointed lecturer in chemistry at Balliol College in 1926. These appointments he relinquished in 1931, when he became senior assistant in biochemistry at the Lister Institute, London, and a reader in biochemistry in the University of London. In 1936, Gulland was appointed to the Sir Jesse Boot Chair of Chemistry at University College, Nottingham.

He gave up this post a few weeks before his death to take up a new appointment as director of research to the Institute of Brewing. He was elected a Fellow of the Royal Society in 1945, and the following year he was appointed a representative of the Royal Society on the British National Committee for Chemistry. He was M.A. of Oxford, Ph.D. of St Andrews, D.Sc. of Edinburgh, and a Fellow of the Royal Society of Edinburgh and the Royal Institute of Chemistry. In 1924 he married Ruth Madeline Ida Russell, also an Edinburgh graduate and daughter of the late Sir James A. Russell, and is survived by his wife and two daughters.

Although he has a large amount of first rate scientific achievement to his credit, Gulland was not quite so prolific a contributor to the original literature as some of his contemporaries. This is partly due to the formidable nature of the problems which he tackled, he never engaged in trivialities. It is probably due also to the fact that his earlier ardour for work at the bench tended to be subordinated later to his interest in organization and administration, for which he had a real flair. He served on the councils of the Chemical Society and the Royal Institute of Chemistry, and was an honorary secretary of the Chemical Society during 1933-6. From 1932 until 1934, he was secretary of Section B of the British Association, and during 1935-7, he was recorder to the Section. In the early years of the Second World War he was senior gas adviser to the North Midland Region of the Ministry of Home Security, and during 1943-4 he was assistant director, Chemical Research and Development, Ministry of Supply. In this capacity he was concerned with the organization and instigation of research and development by firms, research associations, government establishments and universities on a variety of aspects of such topics as general chemicals and explosives, flax, camouflage, adaptation of army equipment of all kinds for use in the tropics, plastics, textiles, paper and rubber. It was in this period also that he was attracted by the potentialities of alginate acid as a chemical raw material, and he took an important part in the establishment of the Scottish Seaweed Research Association. He became a member of its Board of Management, and Chairman of its General Purposes and Chemical Advisory Committees. He was instrumental in forming the Lacc Research Council, of which he was a member, and was an independent member of the Board of Trade Working Party on the Lacc Industry, appointed in 1946.

A full appreciation of Gulland's scientific work cannot be given here and a general indication of some of its main trends must suffice. During his St Andrews and Manchester days, he worked on the morphine group of alkaloids, and the constitution which is now generally accepted for morphine was first adumbrated in two classical papers, published in 1923 and 1925, by Gulland and Robinson. With R. D. Haworth, Gulland published a series of papers on the aporphine group of isoquinoline alkaloids (1928-9). New methods were devised by which were synthesized, among other compounds, bulbocapnine methyl ether and corytuberine dimethyl ether. Gulland also made other chemical studies of compounds of biological interest, including pellitorine, the pungent principle of *Anacyclus pyrethrum*, and the active constituents of *Taxus baccata* (1930-1). His work then followed a more definite biochemical course. With R. A. Peters, in 1929, he investigated the antineuritic properties claimed for certain quinoline and iminazole derivatives and showed that the compounds in question were inactive. Gulland and Peters also showed that pigeons' blood is much richer in reducing substances than is mammalian blood and found that this is due mainly to sulphhydryl compounds, ergothioneine hydrochloride was isolated. In the course of an examination of the spermicidal activity of various quinones and aromatic aldehydes and their derivatives, Gulland found that certain aromatic aldehydes were highly effective spermicidal agents, and he showed how their efficiency could be correlated with some of their chemical reactivities and physico-chemical properties. Between 1932 and 1940, Gulland published a series of papers, in conjunction with T. F. Macrae and other collaborators, on the oxytocic principle of the posterior lobe of the pituitary. He described methods of purification and assay of the hormone, and elucidated something of the chemical nature and active groups by studying the action of nitrous and nitric acids, of oxidizing and reducing agents, and of various preparations of proteolytic enzymes. He was able to show that the oxytocic principle repre-

sents an oxidation-reduction system, but it did not appear to contain peptide linkages. Some synthetic esters of choline had analogous properties, but the activity was very slight.

In 1933 Gulland and Macrae published the first of a series of papers on purine nucleosides. These compounds, with the related nucleic acids, formed the subject of Gulland's main research interests thereafter. His contributions in this important field were noteworthy, not only for their intrinsic value, but also because of the interest which they stimulated in other laboratories. He has himself summarized some of this work in three reviews (Gulland, *J. Chem. Soc.* 1938, p. 1722, 1944, p. 208, Gulland, Barker & Jordan, *Ann. Rev. Biochem.* 1945, 14, 175). By using ultraviolet absorption spectroscopy, Gulland and his collaborators showed the points of attachment of the sugar residues to the bases in the nucleosides, and his conclusions were substantiated by similar measurements on synthetic products by Kenner, Lythgoe and Todd. Again, in the course of a series of investigations on yeast nucleic acid, Gulland (with G. R. Barker) confirmed the identity of the sugar component as D-ribose. Among his last publications was one in this *Journal* (1948, 42, 308) on the nature of the reaction of nucleic acids with mustard gas, and a series of three papers in the *Journal of the Chemical Society* (1947, pp. 1129, 1131, 1141) on the deoxypentose nucleic acid of calf thymus. It is a great loss to British science that his investigations in a field of such supreme biological importance should have been brought to a sad and premature close. It is certain, however, that other workers will come forward to build upon the foundations which he has laid, and there can be no doubt that this is the memorial to his work which he himself would have desired.

(The writer is glad to acknowledge his indebtedness to Mrs J. M. Gulland for some of the personal details, and to the Editors of *Nature* for permission to use as a basis for this tribute a somewhat shorter notice which he contributed to *Nature*.)

J. W. COOK

Studies on the Activation and Purification of Blood Fibrinolysin

By M. ROCHA E SILVA AND C. RIMINGTON

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(Received 26 November 1947)

The presence, in normal plasma or serum, of a fibrinolytic agent that can be activated by chloroform, was first reported by Delezenne & Pozerski (1903 *a, b*). This effect of chloroform was further investigated by Nolf (1905, 1908) who also observed activation of this fibrinolytic capacity of the blood in dogs given peptone intravenously, provided the liver was excluded from circulation either by clamping the thoracic aorta and inferior vena cava ('thoracic animal'), or simply by interrupting the circulation through the liver by manual compression of the vessels going into the organ. More recently, Tagnon (1942) showed that the activity of this fibrinolytic enzyme was connected with the euglobulin fraction of the blood, since it might be fully recovered by isoelectric precipitation (pH 5.5) after dilution of the plasma with 10 vol. of distilled water. A comparison of this fibrinolytic factor with the so-called 'lytic factor' described by Milstone (1941) as the effective fibrinolytic agent of material isolated from certain strains of β haemolytic streptococci (streptokinase), was undertaken by Kaplan (1944), Christensen (1944-5), and Christensen & MacLeod (1944-5). Confirmatory evidence of identity appears in several papers (e.g. Macfarlane & Pilling, 1946; Loomis & Smith, 1946).

Further reports have stressed the importance of the activation of this fibrinolytic enzyme in several physiological and pathological conditions. Using the 'fibrinolytic protamine test', it has been shown (Rocha e Silva, Andrade & Teixeira, 1946) that activation of fibrinolysin occurs in anaphylactic and peptone shock, and that a definite relationship seems to exist between the appearance of the fibrinolytic effect and other characteristics of anaphylactic and peptone shock. In traumatic and burn shock also there is a spontaneous activation of the fibrinolytic enzyme of the blood, the intensity of which bears some relationship to the gravity of the shock condition (Tagnon, Levenson, Davidson & Taylor, 1946). Using normal and sensitized guinea pigs, Ungar (1947) demonstrated activation of a fibrinolytic enzyme when pieces of certain organs (liver and lung) were placed *in vitro* in contact respectively with peptone or with the homologous antigen. Macfarlane & Biggs (1946), using the technique of Macfarlane & Pilling (1946) reported activation of plasma fibrinolysin in a number of subjects from a hospital popu-

lation including cases awaiting operation, under going pretreatment and after actual operation, also some accident and miscellaneous pathological cases. A recent report by Ferguson, Travis & Gerheim (1947) ascribes a weak trypsin like thromboplastic activity to the fibrinolytic enzyme present in normal blood, suggesting participation of this proteolytic enzyme (plasma tryptase) in blood coagulation. The possibility that activation of this enzyme may play a role in the liberation of histamine was recently suggested by Scroggie, Jaques & Rocha e Silva (1947) on the basis of the fact that a trypsin inhibitor from soya beans partially inhibits the release of histamine from cells to plasma when peptone is added to rabbit blood.

The desirability of securing highly active preparations of this fibrinolytic enzyme, in order to study more thoroughly its biological properties, was the main reason for undertaking this study. When the experiments were nearing completion, a paper appeared by Loomis, George & Ryder (1947) in which they described a method for purification of blood fibrinolysin by combining fractionation by ammonium sulphate with several isoelectric precipitations in high dilutions with distilled water. Under present circumstances, we cannot undertake a comparative study of our results with those of the American workers. We have, however, investigated a sample of fibrinolysin supplied by Loomis, and have shown that we can raise its activity appreciably by using a new method of fractionation, as described in the text. Although we did not attain a final purification of the enzyme, the facts described in the present paper may assist a further attack upon the problem.

EXPERIMENTAL

Preparation of material. Ox or human serum or plasma was obtained by prolonged centrifugation of fresh blood. After dilution with 10 vol. of distilled water and adjustment of pH to 5.3-5.5 with dilute acetic acid, the globulin precipitate, which settled overnight in the ice box, was collected by centrifugation and redissolved in one third of the original volume of 1% (w/v) NaCl (subsequently referred to as 'saline') and carefully neutralized with 0.1N NaOH. Chloroform (0.1 vol.) was added and the globulin solution was left for 3 or 4 days at room temperature with occasional shaking. The aqueous layer was then again diluted with distilled water (10 vol.), adjusted to pH 5.3, and the precipitate

allowed to settle in the ice box. It was centrifuged, redissolved in 0.2 of the original volume of saline and neutralized. This 'activated globulin solution' was used for further purification.

Fractionation with ammonium sulphate Appropriate amounts of a saturated solution of $(\text{NH}_4)_2\text{SO}_4$ were added to the 'activated globulin solution'. After standing for 1 hr at room temperature, some 'celite' was added and the precipitate filtered off. The filtrates were dialyzed (cellophane) against several changes of 1% (w/v) solution of NaCl, neutralized and tested for fibrinolysis.

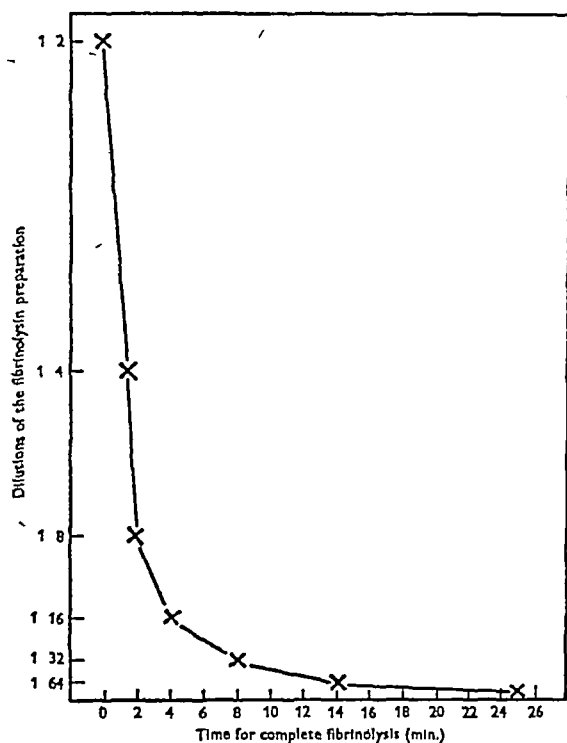


Fig 1 Time dilution curve of the activated ox globulin preparation OxGl_{10} (concentrated 3 times). From data corresponding to dilutions 1.8, 1.16 and 1.32, one can calculate the potency of the preparation as equal to 40 units/ml., $U=10c/t$, where $c=1/D$, D being the dilution, and t the time required for complete fibrinolysis.

'Low ionic' isoelectric precipitation Isoelectric precipitation at low concentration of inorganic ions was performed, as described above, by diluting the activated globulin solution with distilled water (10 vol.) and adjusting to pH 5.0–5.5.

'Saline' isoelectric precipitation The precipitate of the activated globulin solution was dissolved in 1% (w/v) NaCl solution at pH 7.4, acetic acid added to bring the pH to 5.5–5.0 and the precipitate centrifuged and redissolved in neutral saline.

Delipidization of serum or plasma was performed according to the technique of McFarlane (1942) by adding ether (0.3 vol.), shaking and freezing to -25° or -30° with solid CO_2 (dry ice), after slow thawing at room temperature, the mixture was centrifuged and the clear bottom layer was

carefully collected and diluted with 10 vol of distilled water (pH 5.3) and the precipitate collected and treated in the usual way with 0.1 vol of chloroform. As a rule, a duplicate sample of the original serum or plasma (non delipidized) was treated in exactly the same way and the final activity of both samples compared.

Tyrosine was determined according to Folin & Ciocalteu (1927) in 0.1 or 0.2 ml of an appropriate dilution of the material, N NaOH (5 ml), distilled water (4 ml.) and the phenol reagent (1 ml) being successively added. After 20 min standing, the colour was read in a Pulfrich spectrophotometer and the tyrosine concentration determined from a standard curve.

Cholesterol was estimated by the method of Bloor (1928), using the Pulfrich spectrophotometer for the final reading of the colour.

Fibrinolytic activity The fibrinolytic tests were performed in small Wassermann tubes at 37° . To the solution of the enzyme to be tested (0.5 ml), a 0.2% (w/v) solution of human fibrinogen (0.3 ml) and two drops of a thrombin solution (adjusted to produce clot in 1 min) were added. The time for total lysis was measured and the number of units calculated. We found it convenient to define the unit as the amount of enzyme which produces total lysis in 10 min under the conditions stipulated above (cf Fig 1).

RESULTS

Precipitation by ammonium sulphate

The whole of the active enzyme contained in the activated globulin fraction is precipitated between 25 and 37% saturation (room temperature) with ammonium sulphate (Table 1). In a quantitative examination the precipitates at 0–40% and 0–50% saturation with ammonium sulphate were collected, redissolved in saline and dialyzed for 20 hr. The activity of the original solution was almost fully recovered.

Table 1 *Precipitation of fibrinolysin by different concentrations of ammonium sulphate at room temperature*

Materials added (ml)	10	10	10	10	10	10
Activated ox globulin	—	5	6	7	8	10
Ammonium sulphate (saturated solution)	10	5	4	3	2	—
Saline	—	25	30	35	40	50
Conc ammonium sulphate (% satn)	40	42	30	11	—	—
Lytic power of filtrates (units/ml)						

Influence of temperature on the activation of fibrinolysin after removal of the chloroform

The activity of the preparation was very seldom fully developed immediately after the chloroform treatment. Usually, the activity increased slowly, if the preparation was left a few days at room temperature. This spontaneous increase in activity was much slower if the material was kept in the ice box,

and very rapid if maintained at 38° Figs 2 and 3 illustrate such spontaneous increase in activity of the enzyme at 0°, at room temperature and at 38° respectively The shape of the curve in Fig 3 suggests an autocatalytic process, since the increase in activity is slow initially and rapid after 60-90 min

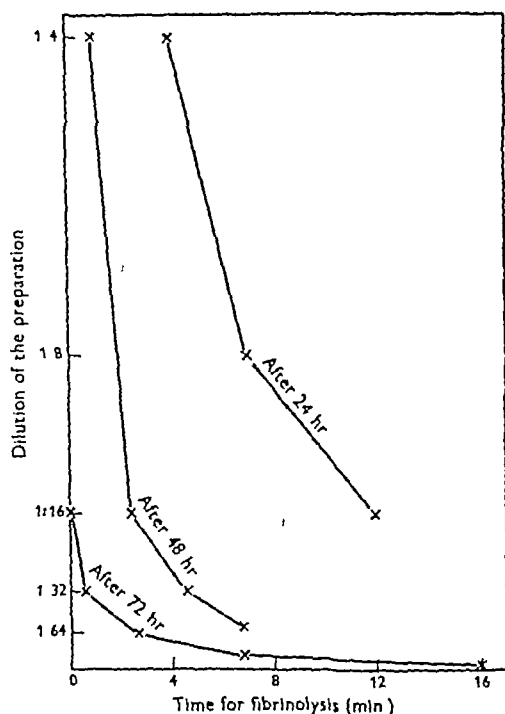


Fig 2 Spontaneous activation at room temperature of preparation OxGI₁₁ after removal of chloroform.

After the maximum is attained, the activity of the enzyme slowly decreases again, possibly owing to the action of the products of the proteolysis As a routine procedure, all preparations, after removal of the chloroform and precipitation at pH 5.3 with 10 vol of distilled water, were maintained for 2-4 hr at 38° We have, however, observed that some of the preparations changed very little after incubation, probably because they were fully activated while in contact with the chloroform

Christensen (1946) demonstrated that chloroform destroys the inhibitor normally present in plasma or serum The shape of the curve of Fig 3 disproves the idea that the further spontaneous activation might

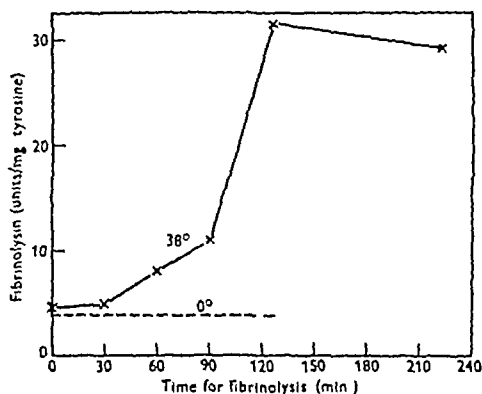


Fig 3 Spontaneous activation at 0 and 38° of preparation OxGI₁₂ after removal of chloroform

be due to a further destruction of the inhibitor, since in that case the maximal increase in activity would occur at the beginning of the incubation That the enzyme is still present in the solution after the chloroform treatment, partially as an inactive precursor (pro fibrinolysin) ready to undergo autocatalytic activation, seems to be the simplest conclusion to be drawn from our study of the effects of temperature upon the activation of the enzyme All attempts to produce a spontaneous activation of the globulin solution without the chloroform treatment, even by incubating the original globulin solution for several hours at 38°, have failed In a few experiments we have tried to activate the globulin solution with carbon tetrachloride and also with ether or ethanol, but in no case was any significant activation detectable by fibrinolysis tests

Isoelectric fractionation in saline

Repeated low ionic precipitation of an activated globulin (AGI) failed to raise its potency appreciably However, by carrying out saline isoelectric pre-

Table 2 Saline isoelectric fractionation of ox globulin preparation OxGI₁₅

Conditions of experiments	Fibrinolysin (units/ml)	Tyrosine (mg/ml)	Fibrinolysin (units/mg tyrosine)
I Solution OxGI ₁₅ , after chloroform treatment	10	2.5	4
II Above, after precipitation with 10 vol. distilled water (pH 5.5) and incubation at 38° (4 hr)	65	2.2	30
III Above, after isoelectric precipitation with 10 vol distilled water ('low ionic isoelectric precipitation')	85	1.7	47
IV Above, after isoelectric precipitation in 1% (w/v) saline (three times) ('saline isoelectric precipitation')	24	0.07	343

cipitation, we obtained a rapid increase in activity/mg tyrosine up to a maximum attained after three or four reprecipitations at pH 5.0-5.3

Table 2 shows the increase in activity of the oxglobulin preparation $OxGl_{15}$, after chloroform treatment, incubation at 38° for 4 hr, reprecipitation at pH 5.5 with 10 vol of distilled water and three reprecipitations in saline, at pH 5.1. A similar experiment with the preparation $OxGl_{16}$, in which incubation at 38° preceded low-ionic isolation precipitation, led to the same result, i.e. a marked increase in potency only when saline was substituted for distilled water

In a few experiments we used human blood, defibrinated or kept citrated. In the latter case, no attempt was made to clot the plasma before the chloroform treatment, but at the end of this treatment the whole fibrinogen was found to be destroyed by the active enzyme. Table 3 shows a protocol of an experiment of isoelectric fractionation of human globulin solution (HGl_2) by three precipitations in saline at pH 5.3

As seen from the examples given, repeated precipitations at pH 5.0 in saline raise the activity to a maximum which may be reached with two precipitations (cf Fig. 4) or may require more. A limitation of this method of purification is that, when the initial activity of the enzyme was low, the maximum attained was also rather low. The only plausible explanation for this is that the inactive form of the enzyme (pro fibrinolysin) is also precipitated in the same range of pH as the active form in such a way that further separation of the two substances by this type of precipitation becomes impossible. Table 4 records all our results with isoelectric precipitation in saline of different preparations of activated ox globulin and one human globulin preparation.

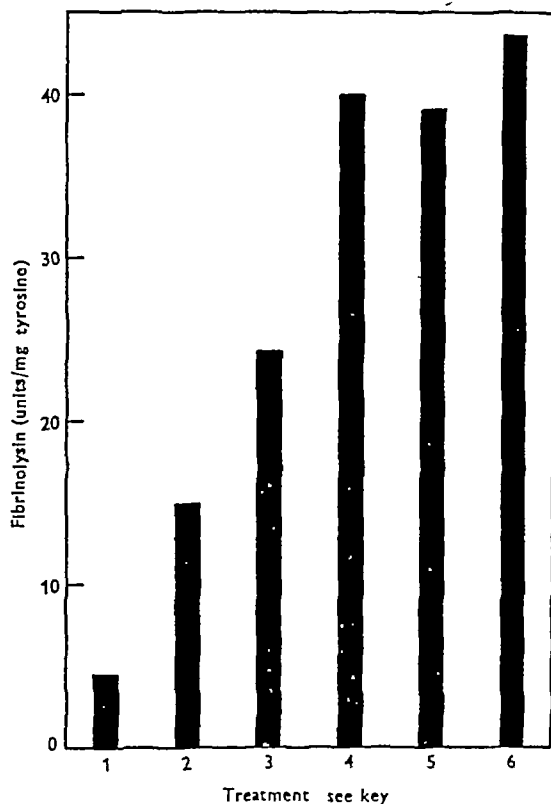


Fig. 4 Activation of preparation $OxGl_{18}$ and progressive purification by isoelectric precipitation in saline. Abscissae: 1, after chloroform treatment, 2, after incubation at 38° for 6 hr, 3, after precipitation in saline at pH 5.0, 4, after repetition of 3, 5, after further repetition of 3, 6, after further repetition of 3.

It is quite clear from Table 4 that, if one starts with a very active preparation, purification by isoelectric precipitation in saline works very well, but

Table 3 Human globulin solution (HGl_2) treated with chloroform and fractionated at pH 5.0 in saline

Conditions of experiment	Fibrinolysin (units/ml)	Tyrosine (mg/ml)	Fibrinolysin (units/mg tyrosine)
I Activated human globulin	60	0.58	110
II Above, after precipitation in saline at pH 5.0	50	0.25	200
III Above, after reprecipitation in saline at pH 5.0	27	0.13	207

Table 4 Increase in activity by fractionation at pH 5.0-5.5 of different globulin preparations

Material	Activity (units/mg tyrosine)	
	After chloroform and incubation at 38°	After isoelectric precipitation in saline (no. of reprecipitation in brackets)
$OxGl_{14}$	17	65 (4)
$OxGl_{15}$	65	343 (3)
$OxGl_{16}$ I	50	132 (3)
$OxGl_{16}$ II	24	158 (3)
$OxGl_{18}$ I	1	9 (4)
$OxGl_{18}$ II	15	48 (3)
Human Gl_2	110	207 (2)
Parke Davis preparation (Loomis <i>et al.</i> 1947)	114	300 (2)

when the initial activity is low, a less satisfactory result is obtained even after several isoelectric precipitations. That this method of purification is different from that recently described by Loomis *et al.* (1947) can be shown by the fact that fractionation in saline at the isoelectric point of a sample of one of the Loomis preparations, increased the specific activity three times. We feel, therefore, that purification by isoelectric precipitation in saline has a useful place, in combination with other methods, in the preparation of purified fibrinolysin.

in the usual way by isoelectric precipitation with 10 vol of distilled water at pH 5.5. The precipitate collected by centrifugation was redissolved in saline, neutralized and submitted to the chloroform treatment, since there was no evidence that the simple treatment of delipidization was enough to activate the enzyme. Simultaneously, a sample of the original serum or plasma was submitted to identical treatment (delipidization excluded) and the final activities of both samples compared. The results (Table 5) show that a previous delipidization of the

Table 5 *Effect of a previous delipidization of serum or plasma by ether at -25° upon the activity of the enzyme resulting from subsequent chloroform treatment*

Exp	Material used	Fibrinolysin		Cholesterol (mg/100 mg tyrosine)
		(units/ml)	(units/mg tyrosine)	
I	DOxGl ₁₁ (delipidized)	15	8.4	14.0
	OxGl ₁₁	1	0.7	2.0
II	DHGl ₄ (delipidized)	33	36.4	17.0
	HGl ₄	20	25.0	9.0
III	DHGl ₅ (delipidized)	55	55.0	12.5
	D ₂ HGl ₅ (twice delipidized)	5	5.0	12.5
	HGl ₅	9	10.0	3.7
	HGl ₅ E (ether saturated)	10	11.0	112.0
IV	DHGl ₃ (delipidized)	40	88.8	8.3
	HGl ₃	33	43.0	4.9
V	DOxGl ₂₀ (delipidized)	12	14.0	28.0
	OxGl ₂₀	0.5	0.5	15.0

Effect of delipidization of the original serum or plasma

The great variability of the results obtained after activation of the serum or plasma with chloroform induced us to study more thoroughly the mechanism by which chloroform activates the fibrinolysin precursor (pro fibrinolysin). Christensen (1946) has shown that chloroform destroys the inhibitor, thus allowing spontaneous activation of the enzyme. It is, however, quite probable that the inhibitor, or at least one of the inhibitors, might be combined with the enzyme precursor. We have assumed as a working hypothesis that this linkage might be of a lipo protein nature, and that chloroform might act by loosening such a linkage. We have, therefore, delipidized serum or plasma in an attempt to activate the enzyme, using first the technique of Hardy & Gardiner (1910) as modified by Hartley (1925). After treatment of the original serum or plasma with a mixture of ether and ethanol at -12° , the material obtained was entirely free from cholesterol or phospholipid, but had no fibrinolytic activity, nor could it be activated by chloroform. We, therefore, decided to try the milder delipidization described by McFarlane (1942), which consists of treating the plasma or serum with ether at -25° or -30° . After this treatment, the globulin fraction was prepared

material definitely increased the fibrinolytic activity after chloroform treatment. In one of the samples of human plasma (D_2 HGl₅), after complete thawing, the underlying plasma was submitted again to the cold ether treatment with a definite decrease in the resulting activity. If one combines this result with that obtained by the Hardy & Gardiner (1910) method, it seems clear that a very mild initial delipidization will improve the final activity, but, if the process of delipidization is repeated, a permanent inactivation of the enzyme occurs.

From the data of Table 5, it might appear that a relationship exists between the final activity of the preparation and its cholesterol content. This seems to be true for most of the experiments in which the untreated material was compared with the delipidized one. In the experiment in which the plasma was saturated with ether (HGl₅E), without freezing the amount of cholesterol present was, however, far greater than that in the delipidized fraction, whereas the final fibrinolytic activity was rather low. It is known that McFarlane's (1942) method does not remove all of the lipids present in serum or plasma. In our experiments 20–40% of the cholesterol was not removed by this treatment. When the globulin fractions were prepared from delipidized serum or plasma, this residual cholesterol appeared to be concentrated in the globulin fraction, very frequently

Table 6 Cholesterol contents of untreated and delipidized plasma or serum and their globulin fractions

Material	Cholesterol content (mg/100 mg of tyrosine)			
	Plasma or serum		Globulin fraction	
	Untreated	Delipidized	Untreated material	Delipidized material
Human P_4	31.0	5.0	9.0	17.0
OxS_{21}	8.7	3.1	2.0	14.0
OxS_{20}	18.0	6.0	15.0	28.0
Human P_3	—	—	4.9	8.3
Human P_5	34.0	7.0	3.7	12.5
DHP_6 (twice delipidized)	34.0	2.5	3.7	12.5

there was more cholesterol in the globulin fraction derived from a delipidized serum or plasma than in that from normal untreated plasma or serum (Table 6). This might be due to the fact that the material, from which the globulin fraction was precipitated, was saturated with ether. In favour of this possibility we might mention the result obtained with the ether-saturated material HGL_6E (Exp. III, Table 5) which contained much more cholesterol than any one of the other fractions obtained from the same human plasma. The results were, however, so paradoxical that a further study of this phenomenon is desirable. For the time being, however, we can accept the explanation that the presence of ether modifies the precipitability of a possible lipoglobulin material that might bear some relationship to the fibrinolytic enzyme.

SUMMARY

1. Experiments on activation and purification of the fibrinolytic enzyme of ox and human plasma or serum showed that the enzyme is associated with the globulin fraction precipitated between 25 and 37% saturation with ammonium sulphate at room temperature.

2. After treatment with chloroform, the enzyme preparation can be further activated by incubation

at 38° for a few hours. The activation process follows the course of an autocatalytic reaction.

3. 'Low ionic isoelectric precipitation' (10 vol of distilled water at pH 5.5) was compared with 'isoelectric fractionation in saline'. The former effected slight purification, whereas the latter rapidly enhanced enzymic potency, indicating that the enzyme is probably connected with a less soluble fraction of the total euglobulin fraction of plasma or serum.

4. A previous mild delipidization of plasma or serum led to a definitely higher activity of the enzyme after the chloroform treatment. A more complete delipidization appeared to destroy the proferment, since no activation could be obtained by subsequent treatment with chloroform.

5. Some relationship has been found between fibrinolytic activity and cholesterol content, especially in those fractions derived from mildly delipidized plasma or serum.

One of us (M. R. e. S.) wishes gratefully to acknowledge the tenure of a British Council Research Fellowship during the course of this work. Our thanks are due to Dr R. A. Kekwick for generous supplies of purified fibrinogen and of thrombin, to our colleague Dr M. Maizels, for arranging the supply of human blood and to Dr C. E. Work for helpful discussions on many problems. Mr J. Evans kindly performed the cholesterol analyses.

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A Study of the Behaviour of some Sixty Amino-acids and other Ninhydrin-reacting Substances on Phenol-'collidine' Filter-paper Chromatograms, with Notes as to the Occurrence of some of them in Biological Fluids

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Although the method of filter paper chromatography (Consden, Gordon & Martin, 1944) can be applied to very many other types of compound (Partridge, 1946, Dent, 1947a, Vischer & Chargaff, 1947, Lugg & Overell, 1947, Maw, 1947), it is likely that its value will continue to be greatest in the amino acid field. In this paper is presented a 'map of the spots' which may be of help to other workers. All the common amino acids and many rare ones have been studied, also a few other interesting substances, some synthetic and not likely to be encountered in nature, which also give a ninhydrin colour reaction resembling that given by α amino acids. Some of the commoner 'unknowns' are also included since they appear to represent new compounds of wide distribution. To include all the 'unknowns' would be difficult, since it has been usual, when working with substances from natural sources, to stumble on them when least expected and in the most bewildering profusion.

EXPERIMENTAL

Points are only raised here which are additional to the earlier recorded work (Consden *et al* 1944, Dent, 1946, 1947a).

Apparatus The cabinet is shown in Fig 1. It is convenient to use and construct. A cabinet of this type has given satisfactory service for two years of almost continuous use. It is much better to have two cabinets, one for the phenol and the other for the collidine runs.

Solvents The phenol must be of A.R. or B.P. quality, too much decomposition with the formation of dirty brown material occurs if cruder phenol is used. An occasional batch of 'pure' phenol has been encountered which has decomposed unduly in spite of all possible precautions. It is a waste of time to persevere with such a solvent.

The nature of the collidine used has been of particular importance. Pure 2,4,6-collidine (2,4,6-trimethylpyridine) obtained from two different American sources completely failed to reproduce the results obtained in England by Consden *et al* (1944) and by the writer, who all used the '2,4,6' compound obtained from a British source. The R_F values were at least half of their reported ones; the insufficient separation of the amino acids which thus ensued making the finished chromatograms almost worthless. On the other hand, a crude American 'collidine' believed to contain very little of the 2,4,6 isomer gave excellent results. By the

kindness of the Reilly Tar and Chemical Corporation samples of pure 2,3, 2,4 and 2,5 lutidines, of 2,4,6 and 2,3,6 collidines, of aldehyde collidine (2-methyl-5-ethylpyridine) and of some crude boiling fractions of pyridine homologues were provided and tried out. The original British results could be well reproduced by taking a mixture of 1 vol. of

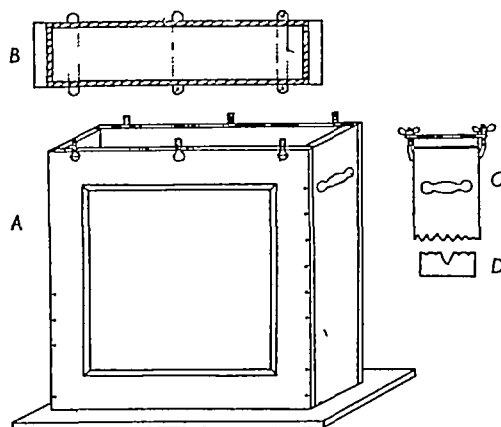


Fig 1. Diagram of cabinet used in two-dimensional chromatography. The cabinet (A) is best made of $\frac{3}{4}$ in. thick 5 ply wood with the sides of one piece. The inside dimensions are 32 x 32 x 8 in. It is provided with a glass window 24 x 24 in. on each side. It must be screwed together firmly owing to a tendency to warp. The inside of the wood is heavily painted with paraffin wax. It is provided inside with a shelf (D) at both ends placed 4 in. from the top; the central notch takes the long glass trough, the lateral ones are used as alternative positions for the glass rods. The under surface of the lid (B) has a rubber gasket which makes an airtight fit against the top edge of the cabinet when screwed down by the wing nuts as in C.

2,4-lutidine and of 1 vol. of 2,4,6-collidine and saturating it at the working temperature with 2 vol. of water. This mixture has been used here and is referred to in future as 'collidine'. This is not claimed to be the best possible mixture, and there is scope for further improvement to meet special points. When pure isomers are not available a crude fraction of pyridine bases boiling around 160° may be adequate. If the R_F values are too large and the spots ill defined in consequence the fraction can be mixed with more

or less of the fraction boiling around 170°. There is some relation between the capacity of the solvent to take up water and its suitability for paper chromatography of amino acids. When 5 ml of water and 5 ml of a good 'collidine' are mixed at about 23°, only about 1 ml of the water remains undissolved. So far, when the water left over measures more than 1.5 ml the solvent has given uselessly low R_F values.

General methods

A variation of more than 5° in temperature during a run is believed to be harmful, at least during the collidine run. The cabinet is therefore always kept, if possible, in an inside room without windows. The operating temperature is, however, less critical than was previously thought. Excellent results have been obtained at room temperatures of 12–31°.

The phenol was always used as the first solvent. Addition of about 0.1 g of NaCN to one of the beakers in the bottom of the box was a great help in preventing oxidation of phenol. Just before closing the cabinet at the beginning of a run, four drops of ammonia solution (sp gr 0.880) were dropped in down the side. Four drops of diethylamine (Consden, Gordon & Martin, 1947) were likewise added just before a 'collidine' run. The sheets were dried at room temperature by directing an electric fan on to them overnight. The ninhydrin reaction was allowed to take place during 24 hr at room temperature (24–30°). This avoids the danger of fading of the colour owing to overheating in the oven. With the exception of α amino isobutyric and of α amino α methylbutyric acids ($q v$) all the amino acids readily develop in this way. No difficulties with the 'pink front' of Consden *et al* (1944) due to contamination by Cu salts have ever been encountered, even when no cyanide was added to the cabinet. Occasional batches of ninhydrin have been purchased that fail completely to give any colours with amino acids.

The amino acids were normally used in the form of a 0.01M solution, 5–10 μ l being nearly always adequate to give a good spot on the paper. The standard solutions are stable over many months if made up in 75% (v/v) ethanol, a small amount of conc HCl being added to dissolve the less soluble ones. When reference is made to the appearance of this or that amino acid in urine or blood it is to be assumed that volumes of 25 μ l of urine and of 125 μ l of protein free plasma have been taken for the chromatogram (see Dent, 1946). There has been no systematic attempt to determine the lower limit of sensitivity for the detection of each amino acid, the figures quoted are therefore usually on the high side. No mention is made as to the optical species, since there is good reason to believe that the active compounds and racemic mixtures travel similarly on the paper. The numerous substances which give very weak colours with ninhydrin are not included in the map, since under the conditions imposed, they are not likely to be present in large enough amounts to be confused with simple amines.

R_F values are not mentioned as such any longer. The rough figures can be surmised from Fig 2 which is drawn to scale. The values in 'collidine' vary so widely with the isomer composition as to be worthless to record. Values in phenol are much more constant, but there is an occasional unaccountable variation of up to 15%, except under carefully controlled conditions, as when two sheets are run simultaneously in the same cabinet. It is stressed that the safest and simplest method of identification, now used exclusively,

is from the pattern of the spots. This is remarkably constant and can be seen at a glance when many amino acids are present together. When only a few are present the most convenient amino acids to add as markers are glycine, alanine, valine and leucine. This extra step is not usually necessary because these same amino acids are the ones most commonly present in greatest amounts in biological fluids.

Special methods

Effect of acidity during the phenol run. The basic amino acids travel much more slowly under conditions of acidity which have little if any effect on the monoaminomono carboxylic acids (Consden *et al* 1944). This is, indeed, a very good test for basicity. The effect is even more marked with more basic substances such as glucosamine ($q v$). Perhaps the best way of carrying this out is to introduce during the phenol run a beaker of 50% (v/v) acetic acid into the cabinet, and, of course, to omit the ammonia. Equal volumes of conc HCl and water may also be used in the beaker, but sometimes this mixture will also slow considerably the monoaminomonocarboxylic acids. The writer can not confirm the claim of Consden *et al* (1944) that the mono aminodicarboxylic acids travel quicker in phenol under acid conditions. Anomalies of their behaviour are discussed further under 'glutamic acid' and 'aspartic acid' in the section on results.

Hydrogen peroxide treatment. This continues to be a valuable technique for investigating the sulphur containing amino acids. The H_2O_2 is applied on the paper itself as previously described (Dent, 1947a), or about 5 μ l may be mixed beforehand with the sample. An interesting point, also discussed later, has arisen with regard to methionine sulfoxide. This substance does not oxidize further to the sulphone under the usual conditions which are adequate to oxidize methionine completely. Indeed, as far as can be judged from the chromatographic method, when a mixture of methionine and methionine sulfoxide is treated with H_2O_2 , the methionine is converted to the sulphone, this presumably having to go through the sulfoxide stage of oxidation, while the sulfoxide which was present from the start remains largely unchanged. Addition, however, of 10–20 μ l of 0.02% ammonium molybdate to the spot on the paper allows the H_2O_2 to convert both methionine and its sulfoxide to the sulphone. There was always increased decomposition of the phenol after H_2O_2 had been used on the paper.

Routine method of investigating a potentially interesting fluid

The following routine is almost unavoidable with a new mixture containing many amino acids, if all of them are to be detected and identified.

(1) Strip method (one dimensional chromatogram) run with phenol. This serves as a rough qualitative and quantitative test. The volume of solution taken for the further tests may have to be adjusted on the basis of this result.

(2) Strip method with 'collidine'. Development is effected with the Pauly diazo reagent (Pauly, 1904) to show histidine and other diazo reacting substances, which may not appear in the later tests owing to their relatively weak ninhydrin colour reactions.

(3) Paper square (two dimensional) run with phenol and 'collidine'. This detects nearly all the amino acids.

(4) Same as (3), but with prior H_2O_2 treatment. This detects cystine and methionine and will also affect other spots if due to dicovalent sulphur compounds (*q v*)

Further chromatograms may be necessary to cover specific points, especially after additions of pure amino acids to act as markers

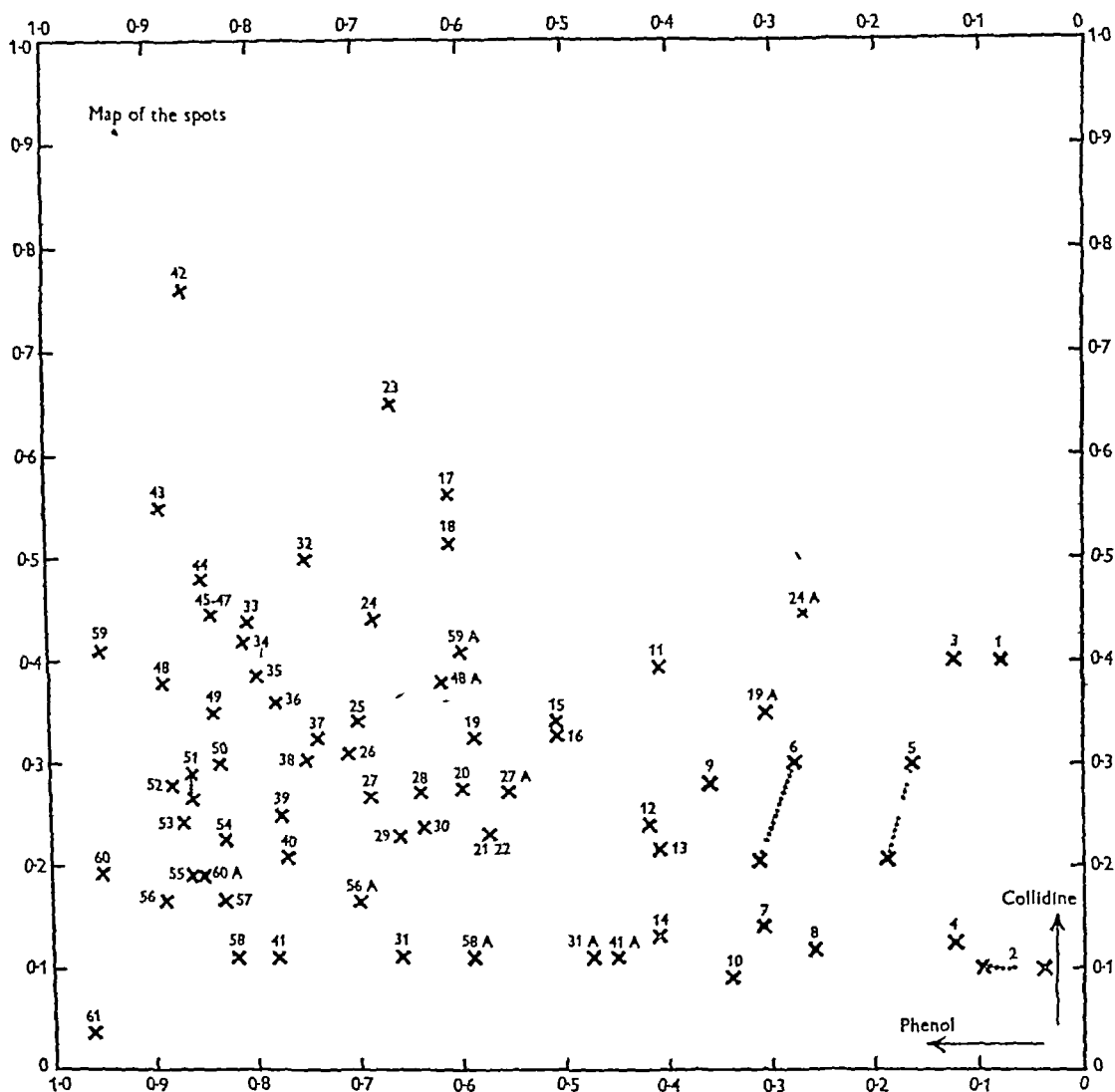


Fig 2 The 'Map of the Spots' The crosses show the average positions taken up on phenol 'collidine' two dimensional chromatograms by the substances in question. See text for the identifications. The map is drawn to scale, assuming that the amino acids start from the point at the right-hand bottom corner, and that the phenol is allowed to soak first along the paper from right to left, as far as the extreme left edge, then the collidine in an upward direction as far as the top edge. Approximate R_F values can be deduced from the scales at the edges. In practice, owing to the lower average R_F values in 'collidine', it is more convenient to run the 'collidine' for a greater distance on the paper than the phenol. This has the effect of spreading the spots upwards to a proportionate extent.

(5) Same as (3), but after hydrolysis with 6N HCl for 24 hr at 100°. The HCl must be removed by evaporation and the solution then made up to its original volume. Spots due to peptides, amides or tryptophan are removed by this process. Amino acids liberated by the hydrolysis appear as new spots or as a strengthening of the spots already given by the unhydrolyzed fluid. It is important to remove traces of protein before the acid hydrolysis.

RESULTS

The behaviour of each amino acid can be seen from the map (Fig 2) and from the summaries below. Substances marked with an asterisk have only been run a few times on the chromatograms, and the results should be accepted with more reserve than

those from the other amino-acids, many of which have been run hundreds of times and under very variable conditions. The position of each substance on the map is shown by the number in parentheses after the name. The colour mentioned refers to the ninhydrin reaction and is to a minor extent dependent on the composition of the 'collidine' used.

Amino-acids or other known substances shown on the map

α-Alanine (20) Detectable in amounts of 2 μg. Gives a purple colour. Nearly always found in urine and other body fluids.

β-Alanine (29) Detectable in amounts of 5 μg. Gives a bluish purple colour. Its position on the map is so close to that occupied by citrulline that there is a serious possibility of confusion, indeed simple position matching has so far always failed to make distinction possible. See 'Citrulline' for a useful spot test. A substance giving a colour in this position is often found in blood, urine and in plant and tissue extracts. It has been proved to be β-alanine in some of these cases.

**alloThreonine* (15) Detectable in amounts of 6 μg. Gives a purple colour. This unnatural amino-acid cannot be distinguished from threonine either in these solvents or in butanol.

α-Amino n-butyric acid (26) Detectable in amounts of 4 μg. Gives a purple colour. This substance has been found in nearly every plant or animal tissue extract so far examined. It is usually present in much smaller amount than its neighbours, alanine and valine. It has not been found in acid hydrolysates of natural proteins, although it has been seen occasionally in the hydrolysates of chemically modified proteins, or in alkaline hydrolysates. Actual isolation in bulk for final confirmation of its structure remains to be done.

**α-Aminoisobutyric acid* (37) Detectable in amounts of 4 μg. Gives a purple colour. The ninhydrin colour reaction occurs much less readily than with α-amino n-butyric acid and the other common α-amino acids. No colour develops after several days at room temperature. It soon appears, however, on heating to 100°. This is presumably due to a steric effect from the fully substituted α-carbon atom (see also α-Amino α-methylbutyric acid). The unusual behaviour with ninhydrin allows of an easy distinction from the isomeric *n*-compound. This acid has not yet been found in nature.

γ-Aminobutyric acid (40) Detectable in amounts of 5 μg. Gives a purple colour. A substance has been found in traces in blood and urine which matches its movements exactly. Both are stable to H₂O₂, to prolonged acid hydrolysis, and are not affected by running the chromatogram at an acid pH during the phenol run. It is probably one of the substances found in fair quantity by Dent, Stepka & Steward

(1947) in potato extracts (no. 23 in their photograph). In the absence of close neighbours to act as reference points it may be confused with the commonly occurring methionine sulfoxide. In such a case, its identity may be confirmed by adding markers or by its stability to H₂O₂ in the presence of ammonium molybdate.

**ε-Aminohexanoic acid* (55) (ε-amino *n*-caproic acid) Detectable in amounts of 5 μg. Gives a purple colour. The ninhydrin reaction occurs as readily as with the α-amino acids. Its position is very different from that occupied by its isomer, norleucine. The acid is not believed to occur in nature.

**α-Amino ε-hydroxycaproic acid* (38) Detectable in amounts of 10 μg. Gives a purple colour. Not believed to occur in nature. A spot in this exact position has, however, been given by the acid hydrolysate of deaminized casein, i.e. casein treated with HNO₂. In the same hydrolysate only a trace of lysine was found.

**α-Aminooctanoic acid* (43) (α-aminocaprylic acid) Detectable in amounts of 6 μg. Gives a purple colour. It has not yet been found in nature.

**δ-Aminopentanoic acid* (57) (δ-amino *n*-valeric acid) Detectable in amounts of 5 μg. Gives a purple colour. The ninhydrin reaction occurs as readily as with the α-amino acids. Its position is very different from that occupied by its isomer norvaline. The acid is not believed to occur in nature.

**α-Aminophenylacetic acid* (33) Detectable in amounts of 8 μg. Gives a purple colour. It is not believed to occur in nature.

Arginine (56) Detectable in amounts of 15 μg. Gives a purple colour. In the presence of 1 l (v/v) acetic acid it takes up the position 56A. The rate of movement in phenol is slowed still further in the presence of 5*N*-HCl. Its presence may be confirmed by means of the Sakaguchi reaction directly applied to the spot on the paper (Consden, Gordon & Martin, 1946a). However, this reaction is of limited value since it is less sensitive than the ninhydrin test and does not work well on spots previously developed with ninhydrin. Arginine is very commonly found in biological fluids.

Asparagine (13) Detectable in amounts of 5 μg. Gives an orange brown colour, sometimes tinged with olive. Although it moves very close to glycine, overlapping always taking place when both are present, it can usually be distinguished by the difference in the colour. Presumably a small amount of asparagine could not be detected in the presence of a large amount of glycine, since the large spot then given by the latter would completely overlap the former. Asparagine has moved rarely to a position slightly above glycine. It has been found in pathological urine, and, of course, in large amounts in plant extracts and also in Amigen—a commercial brand of enzymic casein hydrolysate. It must not be

confused with an unidentified substance, stable to hydrolysis, which also gives a yellow spot and is present in biological extracts. This substance (not shown on the map) moves to a position just below asparagine.

Aspartic acid (5) Detectable in amounts of 5 μ g. Gives a bluish purple colour which develops a little more slowly at room temperature than do the colours of the other common amino acids. The spot usually shows streaking in the direction of the line shown, or may even appear as two distinct spots one over the other. The reason for this is not yet known. The acid is not usually found in normal urine, but often in pathological ones with a high concentration of amino acids (Dent, 1947b). When found in natural sources the spot is often distorted by an unidentified yellow substance which moves between aspartic and glutamic acids.

Urines which do not contain detectable aspartic acid or asparagine often contain large amounts of the former after acid hydrolysis. Presumably the aspartic acid is present in the original urine as a simple derivative, such as an acyl one.

**Carnosine* (54) Detectable in amounts of 15 μ g. Gives a brownish yellow colour which turns greenish if heated to 105°. It has not yet been found in blood or urine.

Citrulline (30) Detectable in amounts of 5 μ g. Gives a reddish purple colour. It may be confused with β alanine which moves to a similar position. It can be distinguished from β alanine by treating the spot on the paper with 5% (w/v) *p* dimethylamino-benzaldehyde in *N*-HCl with which it gives a bright yellow colour, in common with urea and many other urea derivatives, whereas β alanine does not give a colour reaction. Its previous treatment with ninhydrin does not interfere. The aldehyde reaction for citrulline is less sensitive than the ninhydrin colour reaction, hence a negative test must only be considered significant when the ninhydrin reaction is very strong. A weak spot in the position of citrulline and giving the typical reddish purple ninhydrin colour is often seen in normal blood and urine. However, the presence of citrulline in these fluids has not yet been confirmed by the spot test.

Cystathionine (7) Detectable in amounts of 8 μ g. Gives a purple colour. Treatment with H_2O_2 produces a compound which travels more slowly in phenol and decomposes during the collidine run. It has not yet been found in nature by these methods.

Cystic acid (1) Detectable in amounts of 5 μ g. Gives a blue colour. It is formed, apparently quantitatively, from cystine, by treatment with H_2O_2 . Old solutions of cystine may show the presence of cysteic acid. Traces of the latter, occasionally found in urine, may possibly have arisen from cystine by oxidation during storage. Urine from a patient with cystinuria, when treated with H_2O_2 , contains easily

detectable amounts of cysteic acid. This has occurred with the fresh urine as well as with urine that has stood until cystine crystals have deposited. No further evidence has therefore been secured in favour of the theory that the cystine is originally secreted in the form of a complex which decomposes on standing (Brand, Harris & Biloon, 1930). Cysteic acid is found in the hydrolysate of casein treated with H_2O_2 according to the method of Toennies (1942), and also in the hydrolysate of aged wool (Consden *et al.* 1946b).

Cysteine and cystine These compounds are not usually seen in the chromatograms, at least when present in amounts of a few μ g. If large quantities are used, traces of cysteic acid may be detected and also spots due presumably to partial oxidation products. Consden *et al.* (1944), however, report a position for cystine on their chromatograms. The only apparent difference in their working conditions is in the use of the solvents in the reverse order, i.e. 'collidine' first, then phenol.

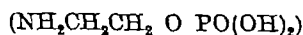
Diiodotyrosine (17) Detectable in amounts of 40 μ g. Gives a dull greenish purple, slightly duller and browner than that given by tyrosine. It readily couples with diazotized sulphanilic acid in Na_2CO_3 solution (Pauly reagent) to give a brownish red azo dye, presumably with the elimination of iodine. Previous development with ninhydrin does not interfere with the diazo reaction.

* $\beta\beta$ -*Dimethylcysteine* (penicillamine) It decomposes on the chromatogram when applied in amounts of a few μ g. Some $\beta\beta\beta'\beta'$ tetramethylcysteine may be formed, however, and is then picked up in its accustomed position (q v).

**Djenholc acid* (14) Detectable in amounts of 5 μ g. Gives a purple colour. Treatment with H_2O_2 produces a compound which travels more slowly in phenol and which decomposes during the collidine run.

Ethanolamine (48) Detectable in amounts of 5 μ g. Gives a purple colour. A serious loss of the material by volatilization always occurs if the paper is dried at 100°, and apparently to some extent when all the operations are carried out at room temperature. When the phenol run is carried out in the presence of 5*N*-HCl the substance finally occupies the position 48A. It is believed that ethanolamine is the substance in pathological urines, previously reported under the name 'fast-valine' (Dent, 1947b). It has also been found in the hydrolysate of gramicidin (Synge, 1945).

Ethanolamine phosphoric acid (10)



Detectable in amounts of 10 μ g. Gives a purple colour, the colour produced at room temperature usually intensifies on heating. It is remarkably stable to acid hydrolysis, plenty of the material

surviving 6N-HCl for 24 hr at 100°. It is unaffected by H_2O_2 . A substance has been found in cancer tissue, intestinal mucosa and body fluids which matches the movement exactly on the chromatogram and also shows the above properties. Ethanolamine phosphoric acid has been isolated from intestinal extracts by Colowick & Cori (1939).

Glucosamine (24) Detectable in amounts of 16 μ g. Gives a purple colour. The ninhydrin reaction occurs as readily as with the common α amino acids. When the phenol run is carried out in the presence of the vapour from 1:1 (v/v) acetic acid it takes up the final position 24A. Glucosamine has been frequently found in the hydrolysates of naturally occurring proteins and is presumably derived from combined polysaccharide.

Glutamic acid (8) Detectable in amounts of 3 μ g. Gives a purple colour. It often occurs as a streak along the direction of the dotted line, the bulk of the colour sometimes being collected at the two ends, almost as if it were trying to form two separate spots (compare the behaviour of aspartic acid). The lower part is always much redder in colour. At other times it may take up an even lower position on the map, thus causing serious danger of confusion with the other amino acids in that region. This very low position is well illustrated in the photograph published by Martin (1947) of the chromatogram given by hydrolyzed gelatine. No reason can be suggested for these unpredictable anomalies of behaviour. Further difficulties suggestive of peptide formation arise when old solutions of glutamic acid are analyzed on the chromatograms. A two year old solution of glutamic acid in 0.4N HCl has appreciable quantities of another ninhydrin reacting substance which moves much more slowly than glutamic acid in phenol and is decomposed back into the latter on acid hydrolysis. Equally old solutions in 75% (v/v) ethanol have given up to four spots widely separated on the paper. The main one of these, which travels to a position between leucine and ethanolamine, is detectable when the solution is only a few days old, and appears to be the γ ethyl ester. Together with other anomalies in the behaviour of glutamic acid already reported in the literature these findings suggest that some fundamental facts in the simple chemistry of the compound still await discovery. Traces of glutamic acid are usually found in blood, urine, and plant tissues. Large amounts may be further liberated by mild acid hydrolysis even when only mere traces of glutamine are present. Presumably the glutamic acid in such cases is present in the form of a simple N derivative, such as an acyl one (compare aspartic acid).

Glutamine (21) Detectable in amounts of 5 μ g. Gives a purple colour. Incorrectly stated to move to the same position as alanine (Dent, 1947a), this substance moves to a position indistinguishable

from that believed also to be occupied by a peptide (see 'Under alanine') containing serine and glycine. Owing to this behaviour the identification of glutamine must always be confirmed by hydrolysis, which should result in the disappearance of the glutamine with simultaneous appearance of at least a corresponding amount of glutamic acid but no increase in the serine or glycine. With this method it now appears that the substance found in this position in normal blood and urine is glutamine and not the peptide 'under-alanine' as previously claimed by the author (Dent, 1947a). Glutamine has also been detected in large amounts in pathological urines, in plant extracts and in Amigen.

***Glutathione (4)** Detectable in amounts of 10 μ g. Gives a purple colour. A spot in this position has been given by tissue extracts. The position given by pure glutathione has not, however, been very exactly reproducible. Further work is required to determine the optimum working conditions for its identification.

Glycine (12) Detectable in amounts of 1 μ g. Gives a slightly reddish purple colour. It is usually the main amino acid in urine, but is usually found in any biological fluid. After its hydrolysis by acid, urine often shows a large increase in glycine, presumably obtained from hippuric acid.

Histamine (59) Detectable in amounts of 16 μ g. Gives a dull greenish purple colour. Its identity can be confirmed by the bright red colour reaction which it gives with the Pauly reagent. The vapour from 1:1 (v/v) acetic acid does not affect its movement in phenol. If 5N HCl be present, however, it is greatly slowed and ends up in the position 59A. It has been found in tissue extracts and possibly in pathological urines. There is a serious danger of losing the histamine owing to its volatility.

Histidine (27) Detectable in amounts of 20 μ g. Gives a dull greenish purple colour. The strength of colour seems to be definitely less than that given by an equimolecular amount of the common α amino acids. Hence the presence of histidine is better detected by means of the Pauly reagent with which it gives an intense red colour. For this test the paper is freed from all traces of phenol or 'collidine' by leaving it overnight in the oven at 100° or by thorough washing with benzene or acetone. Previous use of ninhydrin does not interfere with the Pauly reaction. The position of histidine on the chromatogram varies appreciably with the nature of the 'collidine' used. Pure 2,4,6-collidine brings it to a position well below the level of methionine sulphoxide (no. 39 on the map), and also results in a much browner ninhydrin colour. Some impure 'collidines' of unknown isomer composition may raise it almost to the level of α -amino *n*-butyric acid (no. 26). The movement in phenol is not slowed by the vapour of 1:1 (v/v) acetic acid. It is, however,

slowed to position 27A in the presence of 5N-HCl
Commonly found in biological fluids

**Homocysteic acid* (3) Detectable in amounts of 10 μ g. Gives a blue colour. It is readily formed from homocystine by the action of H_2O_2 . It may be confused with cysteic acid unless careful matching is done. Not yet found in nature.

**Homocystine* This substance does not appear on the chromatogram owing to its decomposition.

Hydroxylysine (31) Detectable in amounts of 15 μ g. Gives a dull purple colour. Its position on the chromatogram agrees with the rule that the addition of a hydroxyl group on to the β carbon atom of an amino acid (lysine in this case) lowers the R_F in phenol with little effect on the R_F in collidine. The amino acid moves to position 31A when the phenol run is carried out in the presence of the vapour from 1:1 (v/v) acetic acid. It has not yet been found chromatographically in natural sources, although the photograph of the chromatogram from hydrolyzed gelatine published by Martin (1947) shows a suspicious smudge in the appropriate position.

Hydroxyproline (28) Detectable in amounts of 15 μ g. Gives a brownish yellow colour. The spot usually touches that of alanine, but the two are easily distinguished by colour. Found a few times in urine, more often in hydrolyzed urines.

**Lanthionine* (8) Detectable in amounts of 6 μ g. Gives a purple colour. Treatment with H_2O_2 produces a compound which travels more slowly in phenol and which decomposes during the collidine run. Not yet found in nature.

Leucine (45) Detectable in amounts of 10 μ g. Gives a purple colour. Coincides with isoleucine and norleucine in the phenol 'collidine' chromatogram. Identification of a spot in this position is therefore best done after isolating the pure amino acid by the cutting out technique (Dent, 1947a, Consden *et al* 1947). The material must then be run on a strip of paper (one dimensional chromatogram) with butanol as solvent. The isomers are readily separated from each other in this way and should be identified by simultaneous comparison with standards of the pure amino acids. Norleucine travels the fastest in butanol and is followed by leucine and then by isoleucine. Alternatively the original chromatogram could be run again with butanol as first solvent in the place of phenol. The positions with this solvent combination have not yet, however, been worked out accurately enough. In the absence of unidentified amino acids whose behaviour in the solvents is unknown, the three leucines can be distinguished by a one dimensional chromatogram run with benzyl alcohol or butanol on the original mixture (Consden, Gordon, Martin, Rosenheim & Synge, 1945). Leucine is commonly found in biological fluids.

Isoleucine (46) Detectable in amounts of 10 μ g. Gives a purple colour. See 'Leucine' for identification. It is commonly found in biological fluids.

Lysine (58) Detectable in amounts of 15 μ g. Gives a dull purple colour. Its spot always tends to be larger than the others and often shows streaking to the right. It takes up position 58A when the phenol run is carried out in the presence of the vapour from 1:1 (v/v) acetic acid. In the presence of 5N-HCl much greater slowing occurs in the phenol. It usually overlaps ornithine if present. For their identification in the presence of each other see 'Ornithine'. Commonly found in biological fluids.

Methionine (34) Detectable in amounts of 10 μ g. Gives a purple colour. Overlaps with leucine, although it may be distinguished when present in high concentration. Its presence is best confirmed by the H_2O_2 technique (*qv*). Methionine slowly oxidizes to the sulfoxide if left to stand some days on the filter paper. It oxidizes more readily during the development of the chromatogram so that a pure sample of methionine usually gives two spots of about equal strength, one for the methionine and the other for its sulfoxide. This artifact of oxidation occurs mainly during the phenol run. The presence of methionine in the original sample may therefore be suspected whenever the sulfoxide is found in its characteristic position (no. 39) (see below for further details). The platinum reagent described by Consden *et al* (1946a) can also be used to detect methionine. The test, however, is less sensitive than the ninhydrin colour reaction and cannot be applied after ninhydrin. It is not positive for the sulfoxide or sulphone, or for cystine. Methionine is only very rarely detectable in biological fluids.

Methionine sulphone (25) Detectable in amounts of 10 μ g. Gives a purple colour. Is formed, apparently quantitatively, by treating methionine with an excess of H_2O_2 . Not believed to occur in biological fluids. It has been found in the hydrolysate of casein treated with H_2O_2 by the method of Toennies (1942).

Methionine sulfoxide (39) Detectable in amounts of 5 μ g. Gives a purple colour. Treatment with H_2O_2 has hardly any effect on it, at most only a trace being further oxidized to the sulphone. In the presence of ammonium molybdate, however, H_2O_2 converts it quantitatively to sulphone. This serves as a most specific confirmatory test of its identity. It is only rarely found in biological fluids. When methionine sulfoxide is found in biological fluids, the question whether it was present as such in the original fluid or was formed from methionine during the phenol run can be settled by treatment with H_2O_2 without a catalyst. The sulfoxide, if present, survives the oxidation and is again found on the chromatogram. If only methionine is present it will all be oxidized to sulphone, and no sulfoxide is found. This

method has been used to confirm the excretion of sulphoxide, as such, in the urine, after giving methionine by mouth (Dent, 1947a)

**α-Methyl α amino n butyric acid* (49) Detectable in amounts of 10 μg Gives a purple colour As with *α aminoisobutyric acid* which is also fully substituted at the *α* carbon, the colour does not develop on standing a few days at room temperature It slowly appears on heating to 100° This behaviour enables it to be easily distinguished from neighbouring substances on the chromatogram It is believed not to occur in nature

Methylhistidine (51) (obtained from anserine) Detectable in amounts of 20 μg Gives a dull greenish purple colour closely matching that given by histidine It does not give any reaction with the Pauly reagent Its spot partly overlaps that of proline from which it is easily distinguished by its colour As shown on the map its relation to proline (no 52) in a vertical direction is subject to a little variation The position slightly above proline is more usual A substance assuming exactly its position and matching its colour with ninhydrin has been found in several pathological urines and has appeared as the strongest spot in the chromatogram from a normal dog's urine

**Monodotyrosine* (23) Detectable in amounts of 30 μg Gives a dull greenish purple colour, closely matching that given by tyrosine It readily reacts with the Pauly reagent to give a brownish red compound indistinguishable from that given by tyrosine Its position is very close to, if not identical with, one of the radioactive spots found by Fink, Dent & Fink (1947) in the chromatogram of the hydrolysate of a rat's thyroid gland removed 24–48 hr after an injection of ¹³¹I, otherwise no evidence has been obtained of its possible occurrence in nature

**Norleucine* (47) Detectable in amounts of 10 μg Gives a purple colour See 'Leucine' for method of identification Not believed to occur in biological fluids

**Norvaline* (35) (*α* amino *n*-valeric acid, *α* amino pentanoic acid) Detectable in amounts of 5 μg Gives a purple colour Not clearly distinguishable from valine, although a spot made up of both valine and norvaline may appear to be double To distinguish between the two the substance giving the spot must be isolated by the cutting out technique (Dent, 1947a, Consden *et al* 1947) and the substance run on a paper strip (one dimensional chromatogram) with butanol This readily separates them, the straight chain compound as usual travelling faster than its branched isomer It is not believed to occur in nature

Ornithine (41) Detectable in amounts of 10 μg Gives a purple colour The spot always overlaps that given by lysine if both substances are present together The two substances appear at first

sight to be giving one large spot The slightly different shades of the colour reaction can, however, usually be observed For final confirmation of identity the chromatogram must be repeated with the vapour of 1 : 1 (v/v) acetic acid present during the phenol run Under these circumstances the much greater slowing of the ornithine (to position 41A) compared with the lysine causes two distinct spots to be produced which are then easily identified Ornithine has not yet been found in blood and urine

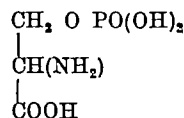
Penicillamine See ββ' dimethylcysteine

Phenylalanine (44) Detectable in amounts of 10 μg Gives a greenish purple colour, with other 'collidines' the colour may be blue or even brownish Addition of a trace of diethylamine to the cabinet is necessary for the phenylalanine to move to the position shown (Consden *et al* 1947) In the absence of diethylamine or if only ammonia is added it invariably moves slower in the collidine and then overlaps the leucines seriously It is often found in pathological urines

Proline (52) Detectable in amounts of 8 μg Gives a lemon yellow colour It is often found in pathological urines

Serine (9) Detectable in amounts of 2 μg Gives a purple colour which may appear brownish when impure 'collidine' is used It is often found in traces in normal urine, much larger amounts may be excreted in disease (Dent, 1947b)

**Serine phosphoric acid* (2)



This was examined in the form of the Ba salt and was detectable in amounts of 10 μg Gives a purple colour It has given either a single or a double spot under apparently similar conditions There is some evidence, however, that the part of the spot moving faster in phenol occurs when the concentration of ammonia is slightly greater It has not yet been found free in nature It is present in a combined state in casein and vitellinic acid

Taurine (11) Detectable in amounts of 3 μg Gives a purple colour The ninhydrin reaction occurs as readily as with the common *α* amino acids Owing to its characteristic position this is a most useful substance to add as a 'marker' to help in the identification of neighbouring amino acids Its position is not altered by the presence of acetic or hydrochloric acids during the phenol run It is commonly found in normal blood and urine In pathological urines it may be excreted in very large amounts to become the strongest spot on the chromatogram This substance was alluded to under the name 'over-glycine' (Dent, 1947a) before it was identified Paper chromatography probably pro

vides the only simple specific test for this widely distributed substance. Many of the methods for determination of amino acids in the urine will include taurine as contributing to the 'total amino-nitrogen'. Nitrous acid and formol methods will do so, and will therefore give quite incorrect results in some pathological urines containing an excess of taurine. The copper method (Albanese, Frankston & Irby, 1944) and the ninhydrin CO_2 method (Van Slyke, MacFadyen & Hamilton, 1943) do not determine taurine.

** $\beta\beta\beta'\beta'$ -Tetramethylecystine (19)* (oxidized penicillamine). Detectable in amounts of 10 μg . Gives a purple colour. Treatment with H_2O_2 and ammonium molybdate causes some of it to be oxidized to an unidentified substance which gives a typical ninhydrin reaction and moves to position 19A. It has not yet been found in biological fluids.

Threonine (16) Detectable in amounts of 10 μg . Gives a purple colour. The colour strength given by this amino acid appears to be weaker than that given by the commoner α amino acids. The vertical position varies slightly with the nature of the 'collidine' used. With some crude batches it has been moved down nearly to the level of alanine. It is commonly found in biological fluids.

Thyroxine (42) Detectable in amounts of 60 μg . Gives an olive brown colour going greener on heating. Thyroxine moves so fast in the collidine that it may run into a brown impurity which usually shows on the finished paper in this region. This impurity is always formed during the phenol run and is subsequently carried forward in the collidine a little way behind its leading edge. When it does this, the thyroxine may be completely lost. There is clearly room for the development of a specific test for this substance which is more sensitive than the ninhydrin reaction, and which gives a colour which will show up against the occasional brown background. So far no success has been obtained in applying the nitrous acid reaction of Kendall & Osterberg (1919).

Tryptophan (32) Detectable in amounts of 20 μg . Gives a purple colour. It is not usually present in blood and urine, although it has been found in the urine of patients with severe liver damage (Dent, 1947b). Not found in acid hydrolysates of proteins owing to its destruction by the acid.

Tyrosine (18) Detectable in amounts of 15 μg . Gives a dull greenish purple colour. Because of the very characteristic and unambiguous position of its spot, tyrosine is a most useful 'marker'. It gives, in similar strength, a brown colour with the Pauly reagent. A sample of 'pure L tyrosine' from a reputable manufacturer was found to contain a large quantity of leucine. It is commonly found in biological fluids.

'Under-alanine' (22) This name was given by Dent (1947a) to a substance present in the urine of

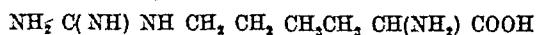
a patient with Fanconi syndrome and cirrhosis of the liver. The substance gave a reddish purple colour with ninhydrin and, on hydrolysis of the urine, the subsequent chromatogram contained no 'under-alanine' and showed a remarkable increase in the content of serine. Experiments on the isolated substance suggested that it might be the tripeptide serylglycylglycine. This substance has not been encountered again, although there is no reason to doubt the original finding. It is now known that both glutamine and a substance in normal blood and urine and in some other pathological urines also move to a very similar position to that of the tripeptide. It is now considered that the substance in normal blood and urine is glutamine and not the tripeptide, as claimed by the writer (Dent, 1947a). It is considered safe to say that a spot in this position is due to glutamine if it disappears on hydrolysis to liberate at least an equivalent amount of glutamic acid without increasing the serine content perceptibly.

Valine (36) Detectable in amounts of 3 μg . Gives a purple colour. Nearly always found in normal and pathological blood and urine and in tissue extracts of plants and animals.

Commonly occurring unidentified substances

'Fast-aminobutyric acid' (50) This is an unidentified substance which has been frequently found in plant extracts and in blood and urine. It is the substance numbered 24 in the photograph of the chromatogram of the potato extract published by Dent *et al* (1947). It is stable to acid hydrolysis and to H_2O_2 and is not slowed during the phenol run by working at an acid pH. It is therefore believed to be a monoaminomonocarboxylic acid. It does not react with diazotized sulphanilic acid or with *p* dimethylaminobenzaldehyde.

'Fast-arginine' (60) This is an unknown substance giving a typical purple colour with ninhydrin. It is stable to acid hydrolysis, and appeared on one occasion to concentrate in a fraction precipitated with phosphotungstic acid. When the phenol run is carried out in the presence of the vapour from 1 l (v/v) acetic acid it is slowed to the position 60A. It is therefore believed to be a new basic amino acid. It has been found several times in blood and tissue extracts and was one of the main amino acids to be passed in the urine of a patient with Fanconi syndrome. Its movement on the chromatogram suggests that it may be the guanidine compound derived from lysine, i.e.



'Green-spot' (53) An unidentified substance giving a bright green colour with ninhydrin. It has been seen in many normal and pathological urines. It survives hydrolysis with 6N-HCl for 24 hr at 100°

'Nephrosis peptide' (61) An unidentified substance found in large amounts in the deproteinized urine in one out of three patients with nephrosis. It was found in the blood of one of the patients who was not passing it in the urine. It gives a typical purple colour with ninhydrin, and on hydrolyzing liberates most of the common amino acids. Traces of a substance moving to a similar position have occasionally been seen in other urines.

Amino-acids not shown on the map

Thiohistidine gives bands in phenol of R_F 0.22. In collidine, however, much decomposition appeared to take place, for the substance which gave a very weak ninhydrin colour at R_F 0.42 no longer gave the Pauly reaction.

Dihydroxyphenylalanine gave bands in phenol and collidine (R_F 0.33 and 0.44 respectively) but has never been seen in a two dimensional chromatogram. Presumably it decomposes during the longer time necessary for this process.

Peptides not shown on the map

Anserine moves readily on the chromatogram to a position near proline. The ninhydrin reaction, however, is so weak that it is unlikely to be picked up unless present in very large amount. Anserine (75 μ g) has been run on a two dimensional chromatogram without being subsequently found. It is more easily seen on one dimensionals. Under such circumstances it gives a dull greenish colour with ninhydrin.

Purely synthetic peptides do not come within the scope of this paper.

Substances that do not react with ninhydrin

Pyrrolidone carboxylic acid does not give a ninhydrin reaction and its movement on the chromatogram has not yet been traced.

This is no place to mention in detail substances which do not react with ninhydrin. It is, however, repeated that the previous suggestion of the writer (Dent, 1947a) that the paper chromatographic method might be of very general application to organic and inorganic substances has been fully confirmed in subsequent trials. The easy separation of chlorides, bromides and iodides by collidine is quoted as a further example of the latter. As a result of this it is useful to bear in mind that the same paper chromatogram which has been run on, say, a specimen of urine, can often, after the amino acids have been identified by development with ninhydrin, be again used for the identification of other substances present, e.g. urea, which moves to a position below tyrosine on the map. There is very great scope therefore for the extension of the

method to simultaneous qualitative analysis covering a wide range of compounds likely to be present in biological fluids.

DISCUSSION

The chief point relative to the more general use of the paper chromatographic method concerns the question of specificity. How far is one justified in assuming that a spot in the exact position of, say, alanine, is in fact due to alanine?

First must be considered the question of the specificity of the ninhydrin colour reaction. There is still much confusion on this subject. For instance, Schmidt (1944) still states erroneously that β and γ amino acids do not give the reaction. As shown here, where only reactions leading to good colour strength are referred to, many primary amines, as well as the β , γ , δ , and ϵ amino acids will give typical reactions, the rate of development of the colour, the strength and the actual shade being identical with that given by α amino acids. However, with mixtures obtained from natural sources only a limited number of such substances is likely to be present. The simple amines are readily detected by subsequent tests depending on the great difference in rate of movement in phenol caused by a difference in acidity (e.g. glucosamine). Substances like taurine may be confused with amino acids, although it seems likely that sulphonic acids will always be detectable by their unusually fast movement in the 'collidine'. The δ - and ϵ amino acids have so far only been reported to be formed as the result of putrefaction (Neuberg, 1911 and Ackermann, 1910, respectively).

Secondly, the question of the accuracy of the position matching arises. Here it must be repeated that there is no need to determine R_F values when enough different amino acids are present to enable identification from the pattern of the chromatogram. With very few exceptions the latter is quite extraordinarily constant, since day to day variables always and variations in the isomer composition of the collidine nearly always affect the R_F values in the same proportions and do not therefore alter the pattern. When a spot giving the typical ninhydrin reaction occurs in the pattern in the exact position of alanine, then the probability is overwhelming that the unknown substance is in fact alanine. A final check must still be done, however, by adding pure alanine to the mixture and then observing that under these identical conditions the pure substance runs exactly with the unknown, and that it is quite stable to hydrolysis. Hydrolysis is most essential, as there are so many possible peptides that every position occupied by an amino acid may presumably be overlapped by unknown peptides. These may give typical ninhydrin reactions but can be decomposed on suitable hydrolysis. For this reason no

certain identification is possible by simple matching of position of substances which are unstable to hydrolysis. The writer's confusion of glutamine with a peptide containing serine and glycine serves as a personal reminder of this fact.

When the unknown spot in the hydrolyzed solution has been matched with that of a known amino acid, the next point to be considered concerns the fact or likelihood of another simple substance moving to the same place. This is a far more serious possibility with amino acids containing more than four carbon atoms (i.e. valine and upwards), since there is a greatly increased chance of isomeric or of totally unrelated substances coinciding. Hence with such substances the identity should be confirmed by isolation by the cutting out technique (Dent, 1947a, Consden *et al* 1947) followed by matching in other solvents. So far, butanol is the only solvent that need be regularly used for this purpose, but the use of any number could be envisaged in case of ambiguity, since it is little trouble to isolate enough for 10-20 trials.

Finally, other tests, more specific than the ninhydrin reaction, can be applied directly to the spot on the paper, as described for histidine, tyrosine and citrulline. Stability to H_2O_2 would also exclude the presence of disvalent sulphur. Basic amino acids are greatly slowed in phenol by acetic or hydrochloric acid vapours. There is much scope for the development of further tests for individual substances.

Bearing all this in mind it must still be admitted that there remains a possibility of confusion between a well known substance and another whose presence has not been suspected and which may happen to coincide on the chromatogram. The risk is believed small and will diminish as our knowledge of natural substances increases.

It will be noted that two dimensional chromatograms are exclusively recommended when a mixture of quite unknown composition is to be analyzed. One dimensionals are only safe when the mixture contains a limited number of amino acids all of known composition and whose behaviour on the paper has already been investigated, as in the analysis of many protein hydrolysates. For several reasons, one dimensional chromatography is not safe in the investigation of the non protein nitrogen fractions of tissues or in the analysis of blood and urine, in all of which new amino acids have been found with embarrassing frequency. First, there is the phenomenon of 'double overlapping', illustrated in Fig 3. Many instances of this have been encountered in practice. Secondly, there is the impossibility when the mixture contains very many amino acids of obtaining good separation of the amino acids from each other on one dimensionals. This may be readily surmised from the published photograph of the two dimensional paper chromatogram obtained by Dent

et al (1947) from an extract of potato tuber. This extract contained at least 24 ninhydrin reacting substances, probably all amino acids. Finally it is often desired to run very large amounts of the mixture on the chromatogram in order to show up constituents present only in relatively small quantities. This can be done on two dimensionals well beyond the point where in one dimensionals the same quantity would show as a continuous streak of colour which would be quite impossible to interpret.

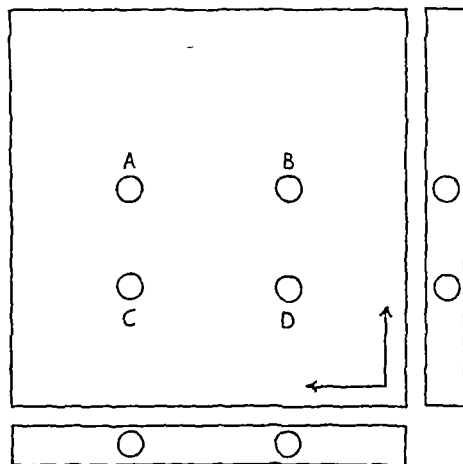


Fig 3 Diagram of paper chromatograms drawn to illustrate the phenomenon of 'double overlapping'. The four circles (A, B, C and D) represent the positions occupied by four hypothetical amino acids after running as usual on the paper square. One dimensional chromatograms in phenol and collidine, run with the solvents in the same directions as on the square, are shown below and to the side respectively. In such a case the square would readily resolve a mixture containing some or all of the substances A, B, C or D. The two one dimensionals if run instead of the square would show no qualitative differences between mixtures containing A and D, B and C, A, B and D, A, C and D, B, A and C, B, D and C, A, B, C and D, since all would appear to contain only two substances. Similar difficulties occur with mixtures only likely to contain three substances, provided that they move to positions similar to three of those in the figure, and in a mixture containing twenty or more amino acids many instances of double overlapping may occur at the same time on different parts of the chromatogram.

The comparison of an unknown substance with a pure compound which is believed to be identical can be made extraordinarily accurate when the latter reference compound can be obtained in radioactive form. The latter is added to the mixture in trace amounts before they are run together on the chromatogram. The final position of the unknown can then be developed as usual with ninhydrin, and the position of the added pure substance determined by means of a radio autograph, i.e. by holding the paper against a photographic plate for a sufficient period of

time. The two compounds are thus developed independently of one another. If they are one and the same they will coincide exactly, not only in their average positions but also in the size and shape of the spot. The slight irregularities which always occur in the shape must be exactly reproducible. The final identification has the finality of a 'finger print' method. This method has been used already by Fink *et al* (1947) in the case of diiodotyrosine containing ^{131}I .

In practice, with substances of natural origin and the solvent combination described here, the only real confusion in the identification of simple amino acids in the absence of peptides occurs in the cases of leucine and isoleucine and of citrulline and β alanine. The methods for distinguishing these are given in the experimental section.

It is conceded that actual isolation in quantity followed by the classical methods of chemical identification should also be undertaken when possible, and is advisable as an independent method of confirmation when claims of the discovery of new amino acids are made. However, it is held strongly that paper chromatography has a place of its own in amino acid problems, at least as important as that of the older methods. The latter are themselves often subject to serious criticism when applied to substances separated from mixtures containing many closely related compounds. Certainly as far as concerns the investigation of mixtures, the chromatographic method is far more specific than any chemical or microbiological method in use at the present time.

Finally, it is urged that a new name be found for a technique which is bound to have wide application as a general method of chemistry and which can only be adequately described now as 'partition chromatography using filter paper'. The name 'papyrography' is submitted for consideration.

SUMMARY

1 The two dimensional filter-paper method of partition chromatography has been applied to some sixty amino acids and other ninhydrin-reacting substances, using the pair of solvents, phenol and 'collidine'.

2 A map is shown giving the positions taken up by these substances on the paper square.

3 With additional tests the method can be applied to the identification of all the ninhydrin reacting substances likely to be present in biological fluids.

4 The ninhydrin colour reaction as used here is positive for many aliphatic amines and also for β , γ , δ , and ϵ amino acids.

5 Peculiarities in the behaviour of glutamic acid, and to a lesser extent of aspartic acid, suggest that there are unknown facts in the chemistry of these substances.

6 Deaminized casein when hydrolyzed contains only a trace of lysine and an ample quantity of α amino ϵ hydroxy-caproic acid.

7 Apart from the common amino acids the following substances have been found in biological fluids by this method of analysis: β alanine, α amino n butyric acid, γ aminobutyric acid, ethanolamine, ethanolamine phosphoric acid, histamine, methionine sulphoxide, methylhistidine. In addition, mention is made of four common but unidentified substances that have been encountered. One ('fast aminobutyric acid') appears to be a monoaminomonocarboxylic acid, another ('fast-arginine') a new basic amino acid.

Grateful thanks are due to the many people in Britain and America, too numerous to mention by name, who have donated some of the very valuable specimens used here, and without whose co-operation this work would have been impossible.

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Some Factors affecting the Extraction of Nitrogenous Materials from Leaves of Various Species

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On the basis of experiments with tobacco, Crook (1946) has worked out a technique for the extraction of nitrogenous materials from leaves. Its usefulness has now been tested on leaves from other plants, although no attempt has been made to apply the technique to a comprehensive selection of plants. Those used had been brought to the laboratory for other studies or were conveniently available from local sources. At the same time it has seemed desirable to review and analyze the data which have accumulated over the last 5 years on the effect of certain factors such as the water and nitrogen contents of the leaves on the extraction of nitrogenous materials from them.

The literature relating to nitrogenous materials of leaves and methods of extracting them has been reviewed by Vickery (1945) and in the previous paper (Crook, 1946). More recently Wildman & Bonner (1947) have described some of the properties of proteins extracted from green leaves by a method differing somewhat from that used here.

EXPERIMENTAL

Leaves Many species were available from the glasshouses. Fig and all cucurbits except marrow, cucumber and bryony were from the University Botanic Garden, Cambridge. The remainder were obtained from plants growing wild or in gardens in the vicinity of Rothamsted. The source is indicated for each species in the tables. No attempt was thus made to control the manurial treatment, age, etc., of the plants, but for certain experiments, advantage was taken of leaf material from fertilizer experiments with tobacco, potato and wheat in which combinations of N, P and K had been applied.

Extraction The general procedure was that outlined by Crook (1946). The leaves were minced in a domestic meat mincer, squeezed through madapolam, re-minced and washed. In certain instances, e.g. strawberry and artichoke, very little or no sap was obtained after mincing. The mince was then washed three or four times with two or three times its weight of water. The washed fibres were then ground in the triple roller mill previously described (Bawden & Pine, 1944) and again extracted with water. Neutralization of fibre to pH 8.0 with 0.2% NaOH was, in most instances, carried out before milling, but it was sometimes convenient to neutralize after milling. N and dry matter extracted during neutralization were included with the mill extract. The leaf fibre of most cucurbits and certain other species

became alkaline on washing (Holden, 1948) and did not require neutralization. Results for these have been listed separately.

Analyses Dry matter determinations were made on portions of extracts by drying overnight at 95–100°. N was determined by a micro Kjeldahl procedure (Crook, 1946).

RESULTS

Extraction of nitrogen and dry matter from various species In Table 1 are summarized the data on the extraction of nitrogen and dry matter from 28 species distributed among 12 families. The disproportionate number of cucurbits (eight species) was included because these plants had been collected to study the 'alkaline drift' phenomenon (Holden, 1948). Species showing this effect are collected together in § (b) of the table, § (a) being for leaves whose fibre requires addition of sodium hydroxide to bring the pH to 8.0.

As was to be expected, the efficiency of the extraction process varies considerably for the different species. Extraction of nitrogen ranges from 93½% with tobacco to 41½% with nettle, and of dry matter from 78% (bryony) to 38% (horse tail). The differences are much more pronounced in mill than in mincer extracts. No consistent differences are noticeable between the two groups of plants. Indeed, § (b) of the table contains the two plants showing greatest and least extraction of nitrogen after milling (bryony and calabash). In general, more nitrogen and dry matter are found in mincer than in mill extracts. Strawberry, laurel, comfrey and bryony are the only species for which this is not true of nitrogen. The last two of these are also exceptional in having more dry matter in their mill extracts than is obtained after mincing.

The ratio of nitrogen in the soluble fraction to that in the material sedimentable from any extract at 3000 r.p.m. may vary widely, for the latter may form a small proportion of the extract as in fig (8½%) or may constitute the major fraction (57%) as in chrysanthemum. However, as there were no obvious regularities in the ratio and as figures for sedimentable material would have complicated the tables unduly, only total extractions have been shown.

The amount of sodium hydroxide required to neutralize the fibre of leaves quoted in § (a) of Table 1 varies considerably, and on this basis the plants can

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Table 1. *Extraction of nitrogen and dry matter from leaves of various species*

Family	Latin name	Common name	Source*	Dry matter (% of wet wt)	N (% of dry matter)	N (% of total)			Dry matter (% of total)		
						In extracts			In extracts		
						Mince	Mull	In residue	Mince	Mull	In residue
(a) Plants whose fibres require neutralization											
Compositae	<i>Senecio vulgaris</i>	Groundsel	W	8.2	4.68	56	36	8	45	26½	28½
	<i>Chrysanthemum horlorum</i>	Chrysanthemum	W	18.6	2.06	31	31	38	41	39	39
Croiflorae	<i>Brassica oleracea</i>	Cabbage	W	0.7	4.47	55	30½	14½	52½	1	34
Rosaceae	<i>Fragaria vesca</i> , var. Mayo	Strawberry	W	33.0	2.84	33	38	29	33	31	36
	var. Royal Sovereign	Strawberry	W	36.1	1.57	39½	—	(60½)	43½	—	(56½)
	<i>Prunus laurocerasus</i>	Laurel	W	28.8	1.75	21	31	48	30	16½	53½
Cucurbitaceae	<i>Echallium elaterium</i>	Squirting cucumber	C	11.8	4.52	47½	42½	10	45½	25	29½
Leguminosae	<i>Phaseolus vulgaris</i>	Bean	G	14.1	2.80	54	19	27	49½	10	40½
Equisetaceae	<i>Equisetum</i> sp.	Horsetail	W	17.1	3.23	37	22	41	25	13	62
Gramineae	<i>Triticum vulgare</i>	Wheat	W	11.0	4.46	67	25	8	47½	17	35½
	<i>Dactylis glomerata</i>	Cocksfoot	W	27.0	2.49	35	34	31	28	15	57
Solanaceae	<i>Physalis alkekengi</i>	Winter cherry	G	23.7	4.03	47	17	36	30	17	53
	<i>Nicotiana glutinosa</i>	—	G	9.8	2.76	58	24	18	61½	12½	26
	<i>Nicotiana tabacum</i>	Tobacco	G	9.5	4.35	51	42½	6½	50	24	26
	<i>Datura stramonium</i>	Thorn apple	G	13.4	4.66	55	38	7	52	27½	20½
	<i>Lycopersicon esculentum</i>	Tomato	W	13.5	3.26	61	22	17	58	15½	26½
Buxaceae	<i>Buxus sempervirens</i>	Box	W	52.1	3.29	45	38	17	31	21	48
Moraceae	<i>Ficus carica</i>	Fig	C	20.2	4.52	50	37½	12½	38	19	43
(b) Self neutralizers											
Compositae	<i>Helianthus annuus</i>	Sunflower	W	17.6	3.24	46	10	44	36	10	45
	<i>H. tuberosus</i>	Artichoke	W	18.9	2.49	35	9½	56½	45	7½	47½
Boraginaceae	<i>Symphylum officinale</i>	Comfrey	W	13.2	4.14	24	28½	47½	21½	42	36½
Urticaceae	<i>Urtica dioica</i>	Nettle	W	21.8	3.75	20	12½	58½	22	8½	60½
Cucurbitaceae	<i>Cucurbita ovifera</i>	Marrow	W	14.7	4.66	53½	21½	25	38½	13	48½
	<i>C. ovifera</i>	Marrow	W	13.2	2.95	56½	12½	31	41	5	54½
	<i>Cucumis sativus</i>	Cucumber	W	9.8	4.38	62½	20½	—	42	17½	43
	<i>Bryonia dioica</i>	Bryony	W	11.1	4.60	36	52½	11½	34	—	—
	<i>B. dioica</i>	Bryony	W	11.5	6.21	40½	48½	11	37	41	22
	<i>Lagenaria leucantha</i>	Calabash	C	11.4	4.21	51½	3½	45	54	3½	42½
	<i>Thladiantha</i> sp.	—	C	13.9	3.17	72	5	23	45	5	50
	<i>Cyclanthera esplodens</i>	—	C	12.0	4.85	58½	28½	13	45½	16	38½

* Source G=glasshouse, C=Cambridge Botanic Garden, W=wild and garden

be divided into two groups. In one are those species, among which is included tobacco, whose fibre takes up moderate amounts of alkali—30–60 ml 0.2N-NaOH/100 g. In the other are those, such as strawberry and chrysanthemum, which take up very large amounts—200 ml/100 g and more. The uptake of alkali in the former, which is due chiefly to the demethylation of pectin by pectase (Holden, 1945), proceeds rapidly at first and then more slowly, and is substantially complete in 24 hr. In the latter group, however, the uptake continues at a steady rate for as long as 6 days. Further, there is a simultaneous darkening in colour which does not occur with tobacco-like species and which suggests that oxidation of phenolic substances is concerned.

Relation between nitrogen and water contents of leaves. A review of the early data extending over the period 25 March 1942 to 26 September 1944 on the nitrogen and dry matter contents of 20 batches of tobacco leaves showed that there was a linear relation between their nitrogen and water contents. In Table 2A are shown the constants for the best straight line through the experimental points calculated by the method of least squares. Application of Fisher's '*t* test' to this data gave a value of $t = 8.86$. The '*t* test' measures the probability of a random set of data conforming to a hypothesis *by chance*, i.e. the probability that the data here presented should fall *by chance* on the calculated line with the present degree of exactitude. The value of *t* here obtained indicates that there is less than 1 chance in 10^8 that this is a random result. Subsequent data for tobacco from three manurial experiments (February and March 1946) treated with all combinations of N, P and K did not give results of such high significance,

t being reduced from 8.86 to 2.62. No explanation for this difference can be given, but it should be noted that, throughout the early experiments, manurial treatment had been 'normal', whereas the later batches included many plants deficient in N or P. This did not appear to affect the other correlations noted below. Twenty batches of potato leaves from manurial experiments showed no relation between the nitrogen and water contents. However, when all species were considered together, a significant relation was again obtained, *t* being such that the probability of a chance result of this type was less than 0.001.

Effect of water and nitrogen content of the leaves on the extraction. Early experiments with tobacco suggested that more nitrogen was extracted from the wetter leaves. This was subsequently confirmed and was also found to hold for all species (Table 2B).

The factor of most significance for the extraction of nitrogenous materials was the initial nitrogen content of the leaves. No difference was detectable between the earlier and more recent data for tobacco, and the high positive correlation between nitrogen content and nitrogen extraction also held for all species together. The correlation was as good for extraction by mincing alone as for those by the whole procedure.

It has been found most convenient to express both extracted nitrogen and total nitrogen as a percentage of the total dry matter of the leaf. On this basis, Fig. 1 has been plotted from the combined data for the tobacco experiments referred to above and is included as an indication of the exactness of these relations. The lines shown are those calculated by the method of least squares.

Table 2 Constants of the regression lines of the form $y = a + bx$ expressing the relations between extraction of nitrogenous materials from leaves and their nitrogen and water contents

Material or fraction	No of exp	a	b		t*
			Value	Standard error	
A y=total N (as % dry matter)					
x=g water/g dry matter					
Early data for tobacco	20	1.39	0.16	±0.02	8.86
Later data for tobacco	24	-1.07	0.57	±0.22	2.62
Data for all species	31	2.33	0.24	±0.06	3.92
B y=extractable N (as % dry matter)					
x=g water/g dry matter					
Tobacco—total extraction	24	-1.78	0.60	±0.20	2.94
All species—total extraction	30	1.20	0.28	±0.07	3.93
C y=extractable N (as % dry matter)					
x=total N (as % dry matter)					
Tobacco Mincer extraction	24	-0.28	0.69	±0.04	16.1
Total extraction	35	-0.34	0.95	±0.04	21.7
All species Mincer extraction	22	-0.45	0.62	±0.08	7.6
Total extraction	31	-1.14	1.06	±0.10	10.1

* Calculated by Fisher's '*t* test'. From this value and the number of experiments can be obtained the probability of the experimental points falling on the calculated straight line by chance. For the number of experiments shown here, values of *t* greater than 2.5 indicate odds of more than 50 to 1 against it being a chance result, and for *t* greater than 3.5 the odds are more than 1000 to 1 against.

In Table 2C are collected the expressions relating extractable to total nitrogen, both for the tobacco data included in Fig 1 and for all species listed in Table 1 together. As can be seen, all are highly significant, the values of t being such that the probability of obtaining any one by chance is much less than 1 in 10^6 .

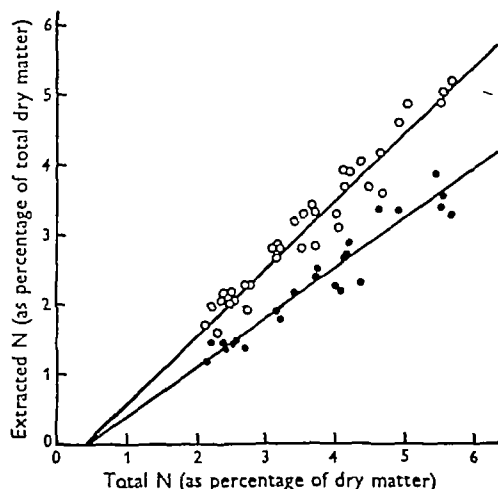


Fig 1 The relationship between total and extractable nitrogen in tobacco leaves ●—● extraction after mincing, ○—○ extraction after mincing and milling

DISCUSSION

There are interesting differences in the ease of handling of various species of plants and in the character of the products. Few regularities of behaviour are observed among related families or even among the members of a single family. In general the wetter and less fibrous leaves are more easily worked although there are important exceptions, e.g. fig and bryony. We have also encountered certain unexplained differences within a single species, e.g. *Ecballium* is given among those plants requiring neutralization. Later samples of this species, however, showed an alkaline drift like other members of the same family.

As a rule, milling is not difficult with any species, provided the fibre is brought to a suitable moisture content. The leaves of some trees are exceptions to this, e.g. those of beech and horse chestnut are not only difficult to mince and give little or no sap on squeezing, but the fibre is so tough and stringy that it will not pass through the triple roller mill. Other plants, such as *Tilia*, contain substances that render their manipulation difficult. This species gives no sap, and, after the addition of water, becomes so slimy and viscous that it is impossible to squeeze extract through the cloths used.

The mincer extracts of several species, e.g. beech, dock and strawberry (var. Royal Sovereign), appear

to be free from protein, i.e. give no precipitate with trichloroacetic acid. Bawden & Kleczkowski (1945) have observed the same phenomenon with this variety of strawberry and ascribed it to precipitation of protein by the large amounts of tannins in the extract. The difference in behaviour of even closely related plants is illustrated by a comparison of the two strawberry varieties. Although the amount of soluble nitrogen in mincer extracts of each is very similar, 53% is precipitable from Mayo extracts with trichloroacetic acid.

From the slope and position of the regression lines of Fig 1 and Table 2C, several conclusions can be drawn. It can be calculated that the average total extraction of nitrogen was 86% from tobacco and 75% from all species. The figure for tobacco is lower than that reported by Crook (1946), but this was based on a procedure involving milling the fibre two or three times. In addition, the average nitrogen content of the leaves on that occasion was rather higher. Both these factors would tend to increase the extraction.

These regressions also make it possible to predict approximately, on the basis of the nitrogen content of the original leaf, the extraction likely to be reached, not only for tobacco, but for any species listed in Table 1. Thus, it can be calculated that, on the average, 78% of the nitrogen can be extracted from a tobacco leaf, 2% of whose dry matter is nitrogen, whereas the extraction rises to 90% for leaves with 6% nitrogen. The differences are greater when the data for all species are considered, the increase being from 48% extraction with leaves containing 2% nitrogen to 86% with those containing 6% nitrogen.

The lines are similar in slope and position both for the extensive data for a single species (tobacco) and for a random sample of plants of various species taken without regard to age, season, manurial treatment or botanical relationship. This perhaps suggests that the observed values are universally applicable and that, if separate regression lines were available for each species, they would all be very similar to that of tobacco.

When extrapolated back, the lines of Table 2C do not pass through the origin, but through points on the total nitrogen axis. This can be regarded as implying that, provided the equation still holds, leaves of this small nitrogen content would show zero extraction, or, what amounts to the same thing, it can be regarded as an illustration of the experimental fact that the last traces of nitrogen are extremely difficult to remove from fibre. As the slopes of the lines for total extraction do not differ significantly from unity, all the nitrogen over and above this limiting quantity is being extracted whatever the initial nitrogen content of the leaf. If the residue could be regarded as 'structural' and unaltered by

grinding and extraction, the picture would be simple—all nitrogen other than this would be extracted. However, the work of Bawden & Pirie (1944) and Crook (1946) suggests that the unextractable residue represents a cross section of the nitrogenous material present in the cells fixed there by the extraction process itself. The plant cell contains a complex mixture of proteins of varying solubility and in various states of dispersion. These might be expected to be extracted at different rates and to different degrees of completeness. It is surprising therefore that extraction from all species of plants should proceed in such a manner that the residue is representative of all proteins and yet at the same time, the degree of extraction is so accurately expressed by a linear regression.

SUMMARY

1 The extraction of nitrogenous materials from the leaves of various plant species has been investigated by a procedure previously worked out.

2 The extraction varied from $41\frac{1}{2}$ to $93\frac{1}{2}$ % of the total leaf nitrogen, the average for all species being 75 %.

3 The extraction was mainly influenced by the nitrogen content of the leaves, increasing with increasing nitrogen content.

4 The extraction was also influenced by the dry-matter content, as the wetter leaves have a higher nitrogen content.

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A Manometric Method for the Estimation of Milligram Quantities of Uronic Acids

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The most widely applicable method for the estimation of uronic acids is that based on the demonstration by Mann (1894) and Mann & Tollens (1896), that uronic acids are decomposed by heating with 12% (w/w) HCl giving a 25% yield of CO₂ (calculated to the anhydride). The method was further explored by Lefèvre (1907) and Lefèvre & Tollens (1907).

In the latter method, the sample was boiled with 12% HCl under reflux for 3.5 hr., and a stream of CO₂-free air passed through the apparatus. After washing and drying, the CO₂ carried from the flask was absorbed in a tube containing KOH. The CO₂ evolved was estimated by the increase in weight of the absorption tube. In subsequent years, many modifications of the original method were introduced, mainly concerned with alterations in the time of decomposition sometimes extended to 5 hr. and even to 8–10 hr. (Ehrlich & Schubert, 1929), and in the methods employed for washing, trapping and estimation of the CO₂. Barium hydroxide became a favoured absorption agent, and titrimetric methods were substituted for gravimetric (cf., for example, Dickson, Otterson & Link, 1930). More recently, attention has been directed to the use of stronger acid to

shorten the time needed for decomposition (Colin & Lemoyne, 1938b, 18% HCl, McCready, Swenson & Maclay, 1946, 19% HCl), and to measurement of rates of CO₂ evolution in order to estimate traces of uronic acid derivatives in the presence of polysaccharides (Norman, 1939; Whistler, Martin & Harris, 1940).

The most radical departure in the determination of uronic acids by means of the CO₂ evolved on decomposition is that of Voss & Pfirsche (1937), who used an approximately 20M solution of ZnCl₂ at its b.p. of 146–147° instead of HCl. All these methods have in common the disadvantage of a complex apparatus and a long decomposition time, usually 5–8 hr.

The method to be described is based on the decomposition of not more than 50 mg. of the sample with 0.25 ml. of 12% HCl in a sealed tube at 111°. The CO₂ evolved is then measured by a modification of the method of Van Slyke & Folch (1940) for manometric carbon determination. The present method has the advantage that samples do not need attention during decomposition, and that, as a consequence, determinations may be made at the rate of 4/hr. for long periods.

METHOD

Preparation of tubes Soda glass tubing, 7 mm external diameter, 5 mm internal diameter, is cut into lengths of 8–10 cm and cleaned by immersion in cleaning mixture for 3 hr. The tubes are washed, rinsed in distilled water, dried, and a bulb of diameter c 15 mm blown on the end of each. If carbonate CO_2 measurements are to be made, the side wall of the tube above the bulb is softened in the flame and a pocket of capacity about 0.5 ml is blown. The tubes are numbered with a commercial black glass marking ink, dried in the oven, and stored in a desiccator. Bulbs prepared in this way have a volume of about 1 ml, and a total volume of about 2 ml after sealing, 60–80 bulbs can be blown/hr.

Weighing of sample The sample should not exceed 50 mg in weight. It must be deposited in the bulb with no contamination of the walls of the tube, otherwise charring will occur on sealing the tube, and extraneous CO_2 will be formed. A convenient spatula for the introduction of solids into the bulb may be made by sealing a V section piece of 'sealing in metal' (obtained from an old radio valve) into a piece of glass tubing that is a sliding fit in the sample tube. If the tubing from which the spatula handle is made fits well, it is almost impossible to deposit material on the walls of the bulb tube if the metal portion is not overloaded. The spatula is introduced holding the tube and spatula horizontal and, when the metal scoop is inside the bulb, it is rotated through 180° , thus depositing the sample, turned to its original position and withdrawn. Liquids are introduced by a capillary pipette. Samples for analysis must be dry. Up to 1 ml of solutions may be dried off in a weighed bulb and then reweighed.

Addition of acid HCl (12% w/w) is used. A pipette is made from the tubing used for the spatula handle. The drawn out tip of the pipette is bent at an angle of 45° from the axis of the pipette, and ground so that it just clears the wall of the tube when the pipette is inserted to the neck of the bulb. This ensures complete delivery and draining of the pipette into the bulb with no loss of acid in the upper parts of the tube. The pipette is fitted with a rubber teat and may conveniently be graduated to deliver four portions of 0.25 ml. If carbonate determinations are being made, the acid is delivered into the side pocket instead of into the bulb.

Decomposition of sample After the addition of the acid, the tube is sealed off by drawing out in the flame, taking care that a narrow prolongation of at least 2 cm is secured. In sealing, 'carbonate' tubes must be held horizontal in order to avoid contact between acid and sample. The sealed tubes are secured vertically in a rack, which is immersed in boiling toluene for 5 hr. The toluene may be boiled in the bottom of a tall beaker, into the upper half of which a condenser made from a loose fitting tin canister is inserted. Such an arrangement permits the toluene to be boiled vigorously for long periods without loss. After 5 hr, the rack and tubes are removed from the beaker and cooled in water. Twenty five samples can be weighed into tubes, the acid added, and the tubes sealed ready for decomposition in 1 hr.

Estimation of liberated CO_2 A sealed tube is inserted as far as the bulb into one end of a piece of pressure tubing c 8 cm long. The other end is attached to the bent tube leading from the gas chamber of the Van Slyke Neill manometric apparatus. The pressure tubing is exhausted twice by lowering the mercury and expelling the air in the usual way.

The second exhaustion serves to verify that there is no leak in the pressure tubing connexion. Two ml of 0.5N NaOH are introduced into the chamber, the mercury lowered to about half way down the large bulb, and the tap turned to connect the bulb to the chamber. The sealed tip of the bulb is then broken by bending the pressure tubing. The subsequent course of the analysis is substantially that described by Van Slyke & Folch (1940). Ten excursions of the mercury are sufficient for transfer of the CO_2 , the process being assisted by tapping the bulb during exhaustion, causing the liquid to coat the walls of the bulb, and warming the bulb in the hand during the mercury upstroke.

Estimation of carbonate CO_2 Samples that contain carbonate are analyzed in the carbonate tubes described above. Two tubes are used for each analysis. In each the HCl is kept from the sample till after sealing, when it is brought into contact with it by bringing to the vertical. One tube is placed in an incubator at 40° for 5 hr and the other in the toluene bath. The percentage carbonate CO_2 obtained by the analysis of the first tube is subtracted from that found for the second to obtain the uronic CO_2 value.

RESULTS

Seven samples of galacturonic acid varying in weight from 4 to 12 mg gave a CO_2 value of 22.33%, s.d. 1.00% (theoretical yield, 22.79%). Since the samples were weighed on a balance reading only to 0.1 mg, these figures show that results are reproducible. Some fifty compounds have been examined for possible interference. The results obtained are listed in Table 1.

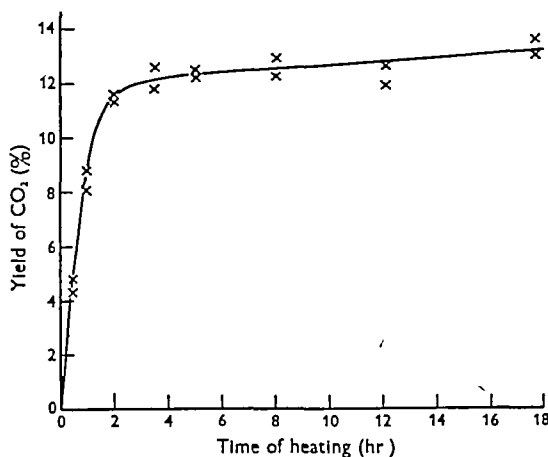


Fig 1 Variation of CO_2 yield with time of heating, citrus pectin

Time of decomposition From Fig 1, which shows the course of CO_2 evolution in the decomposition of a sample of citrus pectin, it is seen that a period of 5 hr appears ample for decomposition, and that the continued CO_2 evolution after 5 hr, due possibly to the decomposition of sugar impurities in the pectin and to the further decomposition of the uronic material, is steady, and in very accurate work can be allowed for (Norman, 1939, Whistler *et al* 1940).

Table 1 *The CO₂ yield of substances tested for interference*

Substance tested	CO ₂ yield (mg CO ₂ /100 mg dry substance)	
	Present paper	Other values
Yields >2%		
Allantoin	cf Fig 2	—
Alloxan	89, 98	—
<i>p</i> Aminobenzoic acid	cf Fig 2	—
Ascorbic acid	171, 187	—
Hypoxanthine	103	—
Pyruvic acid	47	54(2)
Urea	274, 256	—
Yields >1%		
Adenylic acid	18, 19	—
Agar	12	104(1)
Tannic acid	11	—
Tomato bushy stunt virus*	11	—
Yields <1%		
Acetic acid	—	Trace (<0.08)(2)
Amylose (potato)	0.65	—
Arabinose	0.6	0.47(8), c 0.7(11)
Asparagine	<0.1	0(6, 7)
isoBarbituric acid	0.15	—
Caffeine	<0.1	—
Cellulose	0.3	0.45(7), 0.17(8), 0.16(9)
Cholesterol	<0.1	—
Citric acid	0.2	0(6, 7)
Creatinine	0.3	—
Diethylbarbituric acid	0.2	—
Egg albumin	<0.1	0(6, 7)
Ellagic acid	—	0.12(12)
Erythritol	<0.1	—
Formic acid	—	Trace (<0.08)(2)
Fructose	—	0.55(8), 0.80(7), 0.59(9)
Fucose	0.75	—
Fumaric acid	<0.1	—
Galactose	0.5	—
Galic acid	—	0(7)
Gelatin	—	c 0.25(11)
Glucono γ lactone	—	c 0.25(11)
Glucosamine	<0.1	—
Glucose	0.7	0.18(4), 0.73(6, 7), 0.40(8), 0.19(9), 0.26(10), c 0.25(11)
Glutaric acid	<0.1	—
Gluten (wheat)	0.25	—
Glyceric acid	—	0(2)
Glycogen	0.7	—
Glycollic acid	—	0(2)
Glyoxylic acid	—	0(2)
<i>p</i> Hydroxybenzoic acid	—	0.53(12)
Inositol	<0.1	—
Inulin	0.85	0.60(8), c 0.25(11)
Lactic acid	—	0.08(2)
Laevulinic acid	0.2	—
Maleic acid	<0.1	—
Malic acid	0.3	c 0.25(11)
Maltose	—	0.40(8)
Mannitol	—	0(8)
Mannose	—	0.55(8)
Melibiose	0.7	—
Melzitose	0.7	—
2,3,6 Trimethylglucose	—	0.20(8)
Mucic acid	0.3	—
Nicotine	<0.1	—
Nicotinic acid	<0.1	—
Nucleic acid (yeast)	0.75, 0.95	—
Oxalic acid	—	0.42(2), 0.8(3), 2(6, 7), c 0.25(11)
Peptone	—	0.4(7), 0.5(6)
Phenolic acids from redwood bark	—	0.58(12)
Phenylethylbarbituric acid	<0.1	—

Table 1 (cont)

CO₂ yield (mg CO₂/100 mg dry substance)

Substance tested	Present paper	Other values
Yields <1% (cont)		
Rhamnose	—	0.90(8)
Ribose	0.6	—
Salicylic acid	—	0.60(12)
Serine	<0.1	—
Starch	—	—
Potato	—	0.26(5)
Tobacco (pptd. by ethanol)	—	0.57(5)
Tobacco (pptd. by freezing)	—	0.38(5)
Potato	—	0.30(8)
Rice	—	0.45(8)
Wheat	—	0.40(8)
Horse chestnut	—	0.50(8)
Potato	—	0.20(9)
Potato	—	c 0.25(11)
Commercial soluble	0.65	—
Sucrose	—	0.75(7), 0.78(6), 0.52(8), 0.24(9), c 0.25(11)
Tannin (Mimosa)	0.5	—
Tannin (Quebracho)	0.3	—
Tartaric acid	—	0 (6, 7)
Tauroglycollic acid	<0.1	—
Theobromine	<0.1	—
Theophylline	<0.1	—
Thymine	<0.1	—
Tobacco mosaic virus	0.35	—
Trehalose	0.5	—
Uracil	<0.1	—
Uric acid	<0.1	—
Xylose	0.6	0.40(8)

(1) Nanji, Paton & Ling (1925)

(2) Norman & Martin (1930)

(3) Lank (1931)

(4) Anderson (1931)

(5) Spoehr & Milner (1935)

(6) Colin & Lemoyne (1938a)

(7) Colin & Lemoyne (1938b)

(8) Campbell, Hirst & Young (1938)

(9) Norman (1939)

(10) Whistler, Martin & Harris (1940)

(11) McCready, Swenson & MacLay (1946)

(12) Fuller, Bartholomew & Norman (1947)

* Higher value than for tobacco mosaic virus, probably due to carbohydrate contaminant (Bawden & Pirie, 1938)

Interfering substances It has been known for some time that substances other than uronic acids or their derivatives evolve small amounts of CO₂ under the conditions used in the determination of uronic acids. One of the earliest statements is that of Dickson *et al* (1930), who found that the CO₂ yield of various plant acids, sugars, sugar acids, and polysaccharides 'is very small'. Since then, many substances have been tested and the majority have been found to give CO₂ yields negligible for practical purposes (Table 1). A number of substances not previously tested has been found to interfere seriously, however. Some of the previous work has been repeated in order to estimate the magnitude of the effects using the procedure described above, and it will be seen from Table 1 that these effects are of a similar order of magnitude to those found in earlier methods. In addition, other substances have been tested that may be encountered in the analysis of plant material. Some were found to give considerable CO₂ yields under the conditions used. Substances found to give a CO₂ yield over 2% of their weight were *p*-aminobenzoic acid (Weith, 1879),

ascorbic acid, allantoin, alloxan, hypoxanthine, pyruvic acid (Norman & Martin, 1930), and urea

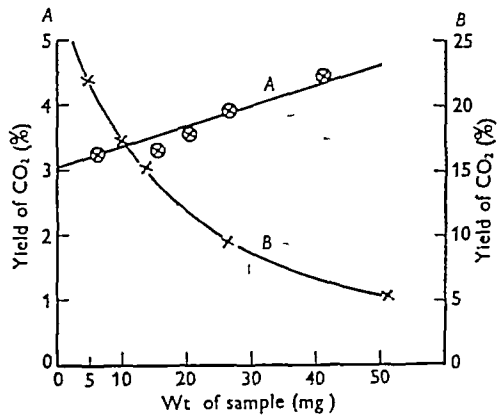


Fig. 2 Variation of CO₂ yield with weight of sample, A, *p*-aminobenzoic acid, B, allantoin

When present in material analyzed by the method described, their CO₂ yield may vary with the amount present. Two examples are given in Fig. 2. If it is

suspected that any of these substances are present it is well to include controls with the amount of interfering substance equivalent to that in the sample. Figures for the percentage of CO_2 given by these substances are included in Table 1.

Since the CO_2 yield of *p* aminobenzoic acid and allantoin varied with the amount used in analysis, it was of interest to determine whether a similar phenomenon was likely to occur with uronic groupings. A series of nine determinations was made on varying weights of the same pectin sample. The average CO_2 yield for five samples (wt 1.3–9.3 mg) was 12.4%, s.d. 1.0%, while that for four samples (wt 13.1–18.2 mg) was 12.0%, s.d. 0.1%. Variation in weight of sample used for analysis does not therefore seem to affect the value obtained materially.

DISCUSSION

The accuracy of the method is perhaps not as high as some of the previous methods, but it is well adapted to the routine analysis of small quantities of material. It has been successfully used in this Station for the routine analysis of samples of plant fibre. The method is not well suited to the study of rates of CO_2 evolution carried out by Norman and

others (Fuller, Bartholomew & Norman, 1947), in order to distinguish between different polyuronides, but these methods have recently been shown not to yield as clear cut results as had been hoped (Fuller, 1947). That a number of substances present in plant, animal, or soil material may interfere to a marked extent is of importance, since it has often been assumed that such interference is unlikely to occur. The interfering substances may assume greater quantitative importance in material that has been fractionated, they might, in fact, be concentrated or introduced during the preparation of fractions.

SUMMARY

1. A rapid method for the analysis of small quantities of materials containing uronic acids or uronides is described.

2. The applicability of the method has been gauged by studying the interference by a number of substances, and results compared with those of previous workers.

3. It has been shown that a number of these substances may interfere seriously with the estimation by the present modification of the Lefèvre & Tollens method.

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The Preparation of Sodium Phosphocreatine

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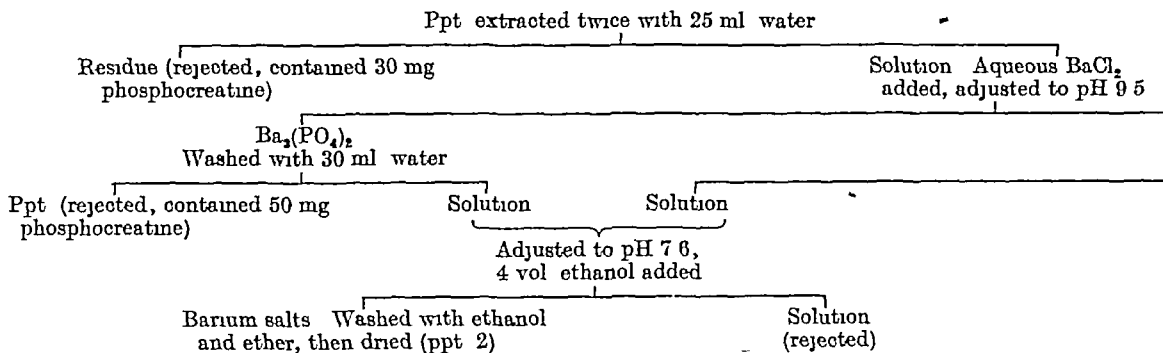
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For an *in vitro* study of the metabolic role of high-energy phosphate compounds it is obviously convenient to employ readily soluble sodium salts. This is especially true where the substance has been isolated as the calcium or barium salt and an excess of the anion or oxalate or sulphate is undesirable. In the case of phosphocreatine there is a further complication since, if the barium salt is used, the precipitation of the barium sulphate results in a loss by adsorption of at least 10 % in solutions containing about 5 mg/ml.

The method usually employed for the synthesis of phosphocreatine is that given by Zeile & Fawaz (1938), but, as these authors point out, many times repeated fractional precipitation is necessary in order to obtain the calcium salt free from chloride. We sought therefore an alternative process for the isolation of pure phosphocreatine simpler than that originally devised by Fiske & Subbarow (1929) and followed in principle by Zeile & Fawaz (1938).

Our method depends on the high solubility of sodium phosphocreatine in water, on the insolubility of barium phosphocreatine in 80 % (v/v) ethanol.

oxychloride (60 ml) and sodium hydroxide (10N) (400 ml) were added to the vigorously stirred solution of sodium creatinate over a period of 2 hr at 0–5°. The product was stirred for 15 min after the last addition and then filtered. The sodium phosphate was washed by stirring with successive amounts of ice cold water (200 and 100 ml), a third extract contained less than 100 mg of phosphocreatine and was discarded. The combined washings and filtrate were adjusted to pH 7.6 with hydrochloric acid (5N) and 3 vol of ethanol (4050 ml) were added. The precipitated salt contained less than 200 mg of phosphocreatine and was rejected. The concentration of ethanol in the filtrate was then increased to 80 % (v/v), this resulted in the precipitation of 36 g of sodium chloride containing an appreciable amount of phosphocreatine. The addition of 1 % (w/v) barium chloride in 80 % (v/v) ethanol to the filtrate precipitated barium phosphate and barium phosphocreatine. The precipitate was separated by centrifugation, washed with ethanol and ether and then dried (ppt 1). The phosphocreatine in the salt was recovered according to the following scheme.



water, and on the fact that sodium and barium chlorides have a solubility of about 1 % in this mixture.

EXPERIMENTAL

Creatine hydrate (20 g) was triturated with 60 ml of 10N sodium hydroxide, 540 ml water added and the insoluble residue removed by filtration. Phosphorus

The combined barium salts (ppts 1 and 2) were extracted three times with 25 ml of water adjusted to pH 9.5 by addition of 0.1N sodium hydroxide. The residue was suspended in 25 ml of water and sufficient hydrochloric acid (N) added at 0° to effect solution. Sodium hydroxide (N) was immediately added to bring the pH to 9.5, and the precipitate of barium phosphate was removed by centrifugation and discarded. The supernatant solution was combined with the original washings and barium pre-

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precipitated by the addition of 10% (w/v) sodium sulphate at pH 7.4. Two washings of the barium sulphate were sufficient to remove most of the adsorbed phosphocreatine, the third extract yielded only 22 mg of phosphocreatine and was therefore rejected. The combined washings and mother liquor now amounted to 220 ml. Ethanol (880 ml) was added and the mixture allowed to stand at room temperature (21°C) for 12 hr to ensure crystallization. The sodium phosphocreatine separates initially in the form of a suspension of very fine oily droplets, and it is advisable to allow the product to stand undisturbed for a considerable time in order to obtain complete crystallization. When this has occurred the mother liquor is perfectly clear and the phosphocreatine remaining in solution is recovered as the barium salt by the addition of 200 ml of 1% (w/v) barium chloride in 80% (v/v) ethanol-water.

The first two preparations we carried out gave a product which crystallized in needles and corresponded to a tetrahydrate, but subsequently we have obtained only the hexahydrate which crystallizes in six-sided platelets. The yield of the sodium salt is consistently 3–4 g and of the barium salt slightly

over 1 g. The combined yield of sodium and barium salts is roughly equivalent to the yield of calcium salt reported by Lehninger (1945). We have not achieved the 30% yield claimed by Zeile & Fawaz (1938). The two hydrates of sodium phosphocreatine were completely free from contamination by sodium chloride and inorganic phosphate.

Sodium phosphocreatine tetrahydrate Found C, 14.6, H, 5.4, N, 12.6, P, 9.7, ash ($\text{Na}_4\text{P}_2\text{O}_7$) 40.4, creatine 38.5, loss on drying at 70° in vacuo 19.2. $\text{C}_4\text{H}_8\text{O}_5\text{N}_3\text{PNa}_2 \cdot 4\text{H}_2\text{O}$ requires C, 14.6, H, 4.9, N, 12.8, P, 10.0, ash, 40.5, creatine 39.9, H_2O , 22.0%.

Sodium phosphocreatine hexahydrate Found C, 13.9, H, 5.4, N, 11.4, P, 8.4, ash, 36.7, creatine 35.3, H_2O , 27.8. $\text{C}_4\text{H}_8\text{O}_5\text{N}_3\text{PNa}_2 \cdot 6\text{H}_2\text{O}$ requires C, 13.2, H, 5.5, N, 11.5, P, 8.5, ash, 36.5, creatine 36.0, H_2O , 29.7%.

SUMMARY

A method is described for the preparation of pure sodium phosphocreatine.

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The Isolation of Coproporphyrin III from *Corynebacterium diphtheriae* Culture Filtrates

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It has been recognized that toxic filtrates of *Corynebacterium diphtheriae* cultures contain a red pigment, and in the routine production of diphtheria prophylactic from such filtrates using the alum precipitate method the final preparation is always coloured pink, unless preliminary steps are taken to remove the pigment. Charcoal has frequently been used as a decolorizing agent, but with culture filtrates from the modification of Mueller's (1939) hydrolyzed casein medium, used at the Wright Fleming Institute for Microbiology for the routine production of diphtheria toxin, the use of charcoal leads to an excessive loss of antigen. Attempts were therefore made to find agents capable of removing the red pigment without serious loss of antigen. A large

number of the more commonly used adsorbents were tested without success, but eventually it was found by one of us (L. B. H.) that aluminium benzoate at pH 6.0 and magnesium hydroxide at pH 9.0 were promising. Of these, the latter was found to be more selective, and in general possessed other properties more suited to the preparation of diphtheria prophylactic. The red pigment thus adsorbed on to the magnesium hydroxide proved to be porphyrin in nature, and was readily purified from the magnesium hydroxide precipitate.

The presence of a porphyrin in toxic filtrates of *C. diphtheriae* cultures was first reported by Campbell Smith (1930), and confirmed by Coulter & Stone (1931) who demonstrated the quantitative relation

between porphyrin and toxin production. Most workers (Dhéré, Glucksmann & Rapetti, 1933, Wadsworth, Crowe & Smith, 1935, Pappenheimer & Johnson, 1937) assumed that the porphyrin was a coproporphyrin, and Jakob (1939) claimed, without giving details, to have isolated coproporphyrin III from *C. diphtheriae* as well as from other organisms. However, in further work by Pappenheimer (1947) it was assumed that the diphtherial porphyrin was haematoporphyrin. It therefore appeared necessary to establish its identity on more certain grounds.

The diphtheria porphyrin has proved to be a mixture of at least three porphyrins, but 98–99% consists of coproporphyrin III, the remainder being uroporphyrin I and a hitherto undescribed porphyrin.

EXPERIMENTAL

The lability of the crude toxin makes it preferable, when preparing the porphyrin as a by-product in the large scale production of diphtheria prophylactic, to perform the magnesium hydroxide precipitation after conversion of the toxin to toxoid.

Preparation of the toxoid. The Park Williams no. 8 strain of *C. diphtheriae* was grown on the casein hydrolysate medium described by Mueller (1939), except that the concentration of nitrogen was reduced to 0.13 g/100 ml (Holt, 1948). After 7 days' growth, the microorganisms were filtered off and the toxin-containing supernatant liquid treated with 0.4% by volume of 40% formaldehyde solution. After standing at room temperature for 2 days, the reaction was adjusted to pH 7.5 with 10N NaOH and the solution incubated at 32° for a further 28 days. The resulting crude toxoid solution was further treated as follows.

Preparation of magnesium hydroxide gel. 250 g (approx. 1 g mol.) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ were dissolved in 5 l. of CO_2 -free water, and to the resulting solution carbonate-free 0.5N NaOH (approx. 4 l.) was added with continuous stirring until the pH was 10. After standing 7–10 days the clear supernatant liquid was decanted from the sedimented gel, the volume of which was now about 2.5 l. (i.e. the gel was approx. 0.4N).

Separation of the porphyrin. 0.4N $\text{Mg}(\text{OH})_2$ gel (6.5 l.) was added with stirring to 130 l. of the crude formal toxoid solution, the pH of which had been adjusted to 8.4 by the addition of about 500 ml 5N NH_3 solution. Spectrophotometric measurement of the original crude toxoid solution showed a content of 5.8 mg porphyrin/l. (equivalent to a total of 755 mg). After standing 1 hr, the $\text{Mg}(\text{OH})_2$, now red in colour, was separated from the supernatant, which contained less than 0.1 mg porphyrin/l., and which was subsequently employed for the preparation of diphtheria prophylactic.

Purification of the porphyrin and preparation of its ester. The red precipitate was suspended in 4 l. of distilled water, and dissolved by the addition of about 500 ml of 10% (w/v) acetic acid, and the pH adjusted to less than 1 by the addition of 500 ml 10N-HCl. After stirring for 10 min the mixture was centrifuged, the purple-red supernatant liquid separated and the residue again extracted by standing overnight with 1 l. of N-HCl. The residual precipitate (now dirty-grey in colour) was centrifuged off, and the supernatant liquid added to that obtained by the first acid extraction.

The united portions (6 l.) were then adjusted to pH 3.3, first by the addition of 50% (w/v) NaOH and finally with 30% (w/v) sodium acetate solution. The porphyrin separated as a brown flocculent precipitate, and after standing overnight the mixture was centrifuged. The supernatant liquid contained 0.375 mg/l. of porphyrin. The precipitate was dissolved in 4 l. 0.5N-HCl, and after standing 24 hr the small brown residue was filtered off and rinsed with a small volume of 0.5N-HCl. The porphyrin was then reprecipitated at pH 3.3–3.4 as before. In all, the porphyrin was precipitated in this way four times at its isoelectric point. Finally, after washing with a little 2% (w/v) acetic acid, the precipitate was dried *in vacuo*. The dried porphyrin was dissolved in 150 ml methanol previously saturated with dry hydrogen chloride. After standing overnight, the deep red solution so obtained was poured into 2 l. of ice water in a large separating funnel, and repeatedly extracted with 150 ml portions of chloroform until the aqueous phase was colourless. The chloroform solution (1200 ml) was successively washed twice with 1 l. quantities of water, once with 1 l. dilute ammonia, and a further three times with 1 l. quantities of water. After filtration through a chloroform-moistened paper, the chloroform was evaporated off leaving the crude ester (578 mg).

Chromatographic purification of the crude ester. The crude ester was dissolved in 200 ml benzene, and poured on to a column of CaCO_3 (4 × 34 cm), and the chromatogram was developed with benzene. The main porphyrin fraction was adsorbed very weakly and readily passed into the eluate, leaving a brown zone and a second red porphyrin zone at the top of the column. On washing the column with chloroform in benzene (1:10 v/v), this second porphyrin zone split into two zones, one of which remained high in the column while the other moved fairly rapidly into the eluate. The remaining porphyrin zone and the brown zone could not be made to pass down the column even with pure chloroform. The adsorbent was allowed to dry, and on extrusion the third porphyrin zone was eluted with large quantities of chloroform containing 1% (v/v) of methanol. The top brown zone was not further examined.

The main benzene eluate was evaporated to dryness, and crystallized twice from chloroform and methanol. 429 mg of coproporphyrin III methyl ester (m.p. 155–157° with a remelt at 181–182°) were obtained. This material in chloroform showed absorption bands at 622.1, 572.0, 533.7 and 502.5 μ , intensity $\text{IV} > \text{III} > \text{II} > \text{I}$ (Hartbridge reversion spectroscopy). A further 50 mg were obtained by working up the mother liquors. After saponification in conc. HCl and dilution to 0.15N in respect of HCl, the spectral absorption curve over the range 400–750 μ was identical with that of coproporphyrin I obtained from the faeces of a patient with congenital porphyria. The Cu complex of the ester was prepared by Fischer's (1937) method, and after five crystallizations gave crystals melting at 218° with absorption maxima at 563 and 526 μ .

Aronoff (1947) has pointed out that the relative intensities of the absorption bands of diphtherial porphyrin in ether, as given in our preliminary communication (Gray & Holt, 1947), are not typical of a porphyrin. We have re-examined the absorption spectrum of our material in ether solution and, although inspection through a hand spectroscope at first sight seemed to confirm our finding, when the spectral absorption curve was plotted with a spectrophotometer, there was no doubt that the relative intensities were in the order $\text{IV} > \text{III} > \text{II} > \text{I}$. It was obvious that with a hand

spectroscope the narrow width of the absorption band in the red led to an overestimate of its intensity. Others have also been misled by the narrowness of the first absorption band, thus Grinstein, Schwartz & Watson (1945) describe the relative intensities of the absorption bands of coproporphyrin I as IV, III, I and II in decreasing order.

The eluate containing the second porphyrin zone was evaporated to dryness, and after recrystallization from chloroform and methanol gave 7.1 mg. of the typical hair-like crystals of uroporphyrin I octamethyl ester (m.p. 288°). In chloroform, this ester showed absorption bands at 625.7, 576.7, 536.5 and 503.6 μ , intensity IV > III > II > I (Hartbridge reversion spectroscopy). The third porphyrin zone has not yet been identified.

DISCUSSION

The isolation of coproporphyrin III ester in relatively enormous quantities in the large scale production of purified diphtheria toxoid provides an abundant and hitherto unexplored source of copro-

porphyrin III, which will be of the greatest value in the investigation of the metabolic relationships of this material in the intact animal. The production of small quantities of uroporphyrin I provides yet another example of the so-called dualism of the porphyrins, attention to which was first drawn by Fischer (1937). In the light of present knowledge it is not possible to speculate on the significance of this dualism to the economy of the organism. The implications suggested by Pappenheimer (1947) regarding the relationship of the production of porphyrin to toxin formation will be the subject of a separate paper to be published elsewhere.

SUMMARY

Coproporphyrin III tetramethyl ester in large yield and uroporphyrin I octamethyl ester in very small yield have been prepared from toxic culture filtrates of *Corynebacterium diphtheriae*.

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Crystalline Bacterial Catalase*

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In recent years, the trend of enzyme research has shifted from the study of the reactions catalyzed to a study of the chemical nature of the enzymes themselves. This has led in the last fifteen years to the isolation of some forty enzymes in a crystalline or highly purified state, and in many cases to the identification of their prosthetic groups, resulting in a completely new outlook on enzyme chemistry.

Little progress, however, has been made in the study of bacterial enzymes from this standpoint, and up to the present there has not been recorded the isolation of a single bacterial enzyme in a pure state. This is the more regrettable since many interesting

enzymes exist in bacteria which have not been found elsewhere, and some bacterial enzymes at least (e.g. lactic dehydrogenase, cytochromes a_1 and a_2) are known to differ greatly from their counterparts in animal tissues. This relative neglect of bacterial enzymes is in part due to purely technical reasons, namely, the difficulties involved in growing the large quantities of bacteria required, and the problem of liberating endo-enzymes from the bacterial cell. The first of these problems is well on its way to solution, but the second is more difficult. Most of the techniques hitherto used for destroying the cell wall and liberating intracellular enzymes (for example, autolysis, vacuum- or acetone drying followed by extraction, shaking with glass beads (Curran & Evans,

* A preliminary account of part of this work appeared in *Nature, Lond.*, 160, 125 (1947).

1942) or grinding with powdered glass (Kalnitsky, Utter & Werkman, 1945), the roller crushing mill (Booth & Green, 1938), ultrasonic disintegration (Stumpf, Green & Smith, 1946, and others)) either tend to destroy labile enzymes, are difficult to employ on a large scale, or require specialized apparatus.

This paper describes the use of a relatively little-used method of liberating enzymes from bacterial cells, namely, lysis of the bacteria with lysozyme. This substance, which is now easily prepared in crystalline form from egg white, rapidly brings about smooth and complete lysis of susceptible bacteria, no specialized apparatus is required, and the method can be employed on any scale. Penrose & Quastel (1930), Quastel (1937) and Epps & Gale (1942) have used lysozyme to determine the true enzyme content of bacterial cells grown under different conditions, but it has not previously been used as an aid to enzyme purification.

The present paper describes the application of this method to the isolation of catalase from *Micrococcus lysodeikticus*. Catalase was one of the first enzymes to be described in bacteria (Gottstein, 1893) and much work has been done on it since, but no attempts have been made to isolate it in a pure state or determine its chemical nature. Using lysozyme to liberate the enzyme from the bacteria, we have been able to isolate *M. lysodeikticus* catalase as a pure, crystalline protein. As far as we are aware, this is the first bacterial enzyme to have been crystallized.

Bacterial catalase is on the whole very similar to the catalases that have been isolated from mammalian tissues, but there are certain differences. It is a conjugated protein with haematin as the prosthetic group, but unlike liver catalase it contains no verdohaematin. The protein part of the molecule has the same molecular weight as liver catalase protein, but differs in its resistance to organic solvents and low pH. The catalytic activity of the bacterial enzyme is considerably higher than that of blood or liver catalases, this difference also is to be attributed to the protein part of the molecule. Finally, it differs from other catalases in its crystalline form.

METHODS

Bacterial suspensions The strain of *M. lysodeikticus* used (National Collection of Type Cultures no. 2685) was a descendant of that originally isolated by Fleming (1922). For large scale production, the bacteria were grown on C.C.Y. lactate agar (Gladstone & Fildes, 1940) in enamelled trays (photographic developing dishes) measuring 11 × 16 in., with overlapping lids, each containing 500 ml. of agar. Three ml. of inoculum, prepared by suspending the 24 hr. growth from a Roux bottle in 50 ml. of saline, was spread evenly over the surface of each tray with a glass spreader, and the trays incubated at 35°. Growth was almost complete in 24 hr., but the catalase activity of the bacteria continued to increase

up to 40 hr., which was therefore adopted as the standard time for incubation. The bacterial growth was then removed from the agar surface with a scraper. The average yield was c. 1.5 g. bacteria (dry wt.)/tray.

Estimation of catalase activity The activity of catalase preparations was measured essentially according to Euler & Josephson (1927). Suitably diluted catalase solution (1 ml., containing c. 0.3 μg. of pure enzyme) is added to 49 ml. of 0.015N H₂O₂ in 0.015M phosphate buffer of pH 6.8, kept at 0° in an ice water bath. Five ml. are immediately withdrawn, pipetted into 5 ml. of 2N H₂SO₄, and titrated with 0.01N KMnO₄. Further samples are withdrawn and titrated after 3, 6, 9 and 12 min. For each time interval, the first order velocity constant is calculated as $k = \frac{1}{t} \log_{10} \frac{a}{a-x}$, where a is the initial KMnO₄ titre and $a-x$ the titre after t min.

Under these conditions the reaction is approximately first order but, as with catalase of plant and animal origin, significant destruction of the enzyme by the H₂O₂ occurs, causing falling values of k . Following Sumner (1941), we plot k against t and determine k_0 , the value of k at zero time, by extrapolation. The amount of catalase taken should be such that k_0 falls between 0.025 and 0.04 min⁻¹, the value of k_0 is then directly proportional to the amount of enzyme taken.

Catalase solutions always have to be highly diluted for testing, the activity of the undiluted solution is calculated by multiplying the value of k found in the test by the overall dilution factor, e.g. if 1 ml. of a 1/10,000 dilution gives a k value of 0.03 in the test, then k for the undiluted solution is 0.03 × 50 × 10,000 = 15,000. The purity of an enzyme preparation is expressed according to Euler & Josephson (1927) as $Kat f = \frac{k}{g \text{ enzyme in 50 ml.}}$, or under the above test conditions, $Kat f = \frac{k \text{ in test}}{g \text{ enzyme in test}}$.

To obtain reliable results by this method, it is essential that all glassware should be cleaned with H₂SO₄, K₂Cr₂O₇, and that the very dilute enzyme solutions necessary for the test should be used immediately after diluting. Even so, we consider the error to be about 5%. All measurements reported in this paper are the means of duplicates.

Dry weights Well dialyzed enzyme solutions, or bacterial suspensions thoroughly washed in distilled water, were dried to constant weight at 105° and weighed on a micro balance. The colorimetric method of Pressman (1943) was used as a rapid, rough method of determining the protein content of enzyme fractions, all measurements reported, however, are based on direct weighings. Dry weights of bacterial suspensions were as a routine measured turbidimetrically using a Hilger photoelectric absorptiometer previously calibrated with suspensions of known dry weight.

Haemin estimations The method chiefly used was the pyridine haemochromogen method of Keilin & Hartree (1936) and Rimington (1942). Results were occasionally checked by the cyanhaematin method of King & Gilchrist (1947), the two methods gave identical results.

Crystalline lysozyme This was initially prepared from egg white by bentonite adsorption according to Alderton, Ward & Fevold (1945). Once a supply of seed crystals had been prepared, subsequent batches were made by direct crystallization from egg white (Alderton & Fevold, 1946), this method was found very simple and reliable.

Absorption spectra were observed with a Hartridge reversion spectroscope (calibrated with a neon lamp), and a Beckman spectrophotometer

RESULTS

Action of lysozyme on Micrococcus lysodeikticus suspensions

When a dilute suspension (say 1 mg/ml) of *M. lysodeikticus* in 0.5% saline is treated with a small amount of lysozyme, the turbidity rapidly disappears leaving an almost water clear solution in which no intact bacteria are observable microscopically, the phenomenon has been described in detail by Fleming (1922, 1929) and subsequent workers

An interesting finding is that the catalase activity of *M. lysodeikticus* suspensions invariably increases after lysis with lysozyme, usually by about tenfold. For example, a typical suspension gave the following results (tested at 30°)

	Bacteria in test (mg)	<i>k</i>	<i>Kat f</i>
Before lysis	0.424	0.0382	89
After lysis	0.071	0.0606	854

The same effect is observed if the bacteria are disintegrated by shaking with glass beads. Similar results were observed by Penrose & Quastel (1930), and Krampitz & Werkman (1941) found that intact *M. lysodeikticus* suspensions had no action on oxaloacetic acid, whilst lyzed or acetone-dried bacteria rapidly decarboxylated this substance. The simplest explanation is that diffusion of substrate into the cell is a limiting factor

Fleming (1929) noted that high salt concentrations inhibit lysis by lysozyme. We find also that in the total absence of salts (bacteria washed with distilled water and treated with dialyzed lysozyme solutions) lysis is almost completely inhibited for prolonged periods. The optimal salt concentration is about 0.5% for NaCl and 0.02–0.05M for phosphate buffers, under these conditions, 1 mg of crystalline lysozyme is more than sufficient to bring about complete lysis of 1 g of *M. lysodeikticus* in 1 hr at 30°

Previous workers on lysozyme have only studied its action on quite dilute bacterial suspensions. When large amounts of bacteria in thick (1–8% dry weight) suspension are lyzed, interesting new phenomena are observed. When acted on by lysozyme, such suspensions of *M. lysodeikticus*, which are originally canary yellow and completely opaque, rapidly become greenish yellow and semi-transparent though not completely water clear, the residual turbidity is due to the high concentration of 'ghosts'. As these changes take place the initially mobile suspension becomes highly viscous and slimy. The solution produced by lysis of a 1% bacterial suspension has

about the consistency of egg white, while an 8% solution is a semi-solid gel which cannot be pipetted or poured out of a test tube, a 4% solution is the strongest that can conveniently be handled. Besides being highly viscous, such solutions have marked elastic properties. They are readily drawn out into threads, and on pouring from a measuring cylinder emerge as an elongated 'blob' which slowly descends while the cylinder is tilted and runs back into the cylinder if the latter is restored to an upright position. When caused to flow along a 2 mm horizontal capillary under pressure (in an apparatus similar to that used by Scott Blair, Folley, Malpress & Coppen (1941) for testing the flow elasticity of bovine cervical mucus), they show marked recoil when the pressure is suddenly released.

The chemical nature of this viscous substance or substances is unknown, nor is it known whether they are initially present inside the bacterial cell and released from it by lysis, or formed by the action of lysozyme, it is hoped to investigate these problems in the future. The phenomenon is not peculiar to *M. lysodeikticus*, similar viscous substances being formed when other sensitive bacteria are treated with lysozyme.

Isolation of crystalline catalase from lysed bacteria

The essentials of the purification method we have adopted are as follows. The solution of lyzed bacteria is treated with 0.5 vol of ethanol at pH 5.6, which precipitates the viscous substances formed on lysis, and then shaken with chloroform, which denatures considerable quantities of inert proteins. The aqueous ethanolic solution is then treated with solid ammonium sulphate which, with correct proportions of the three components, causes it to separate into two liquid phases, one containing all the catalase while the other contains most of the contaminating proteins. This 'partition' method is, as far as we know, a novel procedure in protein purification, and may well be applicable to other problems.

After repetition of the above process, two fractionations with ammonium sulphate bring the enzyme to 70–80% purity, when it can be crystallized either by prolonged dialysis or by careful addition of ammonium sulphate.

The following are the details of a typical preparation.

Stage 1. The bacterial growth from 156 trays (78 l of medium) was harvested, suspended without washing in c. 3 l of 0.5% NaCl, and strained through muslin to remove flakes of agar. Turbidimetric estimation showed that the total dry weight of bacteria was 203 g, and 0.5% NaCl was added to make the final concentration of the suspension 4% on a dry weight basis (5085 ml). Crystalline lysozyme was then added (1 mg/g bacteria) and the suspension incubated 1 hr at 30°, when lysis was complete. *Kat f* of lyzed suspension = 910.

Stage 2 The highly viscous lyzed suspension was treated successively with 609 ml of M acetate buffer of pH 5.6, and 2800 ml of ethanol (final ethanol concentration 33.3% v/v). A bulky yellow gelatinous precipitate was centrifuged off, washed with 2500 ml of M/15 acetate (pH 5.6) containing 33.3% ethanol, and the washings added to the original supernatant fluid. This gave 8180 ml of a pale yellow mobile liquid, *Kat f* = 4020. (All the above operations were carried out at 0°, this is not absolutely essential, but gives better yields.)

Stage 3 The above liquid was treated with 1820 ml of chloroform, shaken on a fast mechanical shaker for 15 min and centrifuged, when two liquid layers formed with a thick layer of denatured protein at the interface. The top layer (7610 ml) was siphoned off, *Kat f* = 5200.

Stage 4 The top layer from the last stage was treated with $\frac{1}{10}$ vol of M sodium acetate and 2400 g of solid $(\text{NH}_4)_2\text{SO}_4$ added (30 g to each 100 ml). This caused the separation on standing of two liquid layers, the lower containing most of the $(\text{NH}_4)_2\text{SO}_4$ and the upper most of the ethanol, some $(\text{NH}_4)_2\text{SO}_4$ and water. The smaller top layer (2760 ml) was pale brown and contained all the catalase, whose characteristic absorption band at $631\text{ m}\mu$ could now be seen with a hand spectroscope, *Kat f* = 15,650. (It is essential for the success of this step that the proportions of aqueous solution, ethanol and chloroform specified in stages 2 and 3 should be adhered to, otherwise two layers may not separate, or the catalase may be precipitated at the interface. The sodium acetate is added to keep the pH at about 5.6 on addition of the $(\text{NH}_4)_2\text{SO}_4$.)

Stage 5 The top layer from the above stage was shaken for 15 min with an equal volume of chloroform, allowed to stand, and the top layer (1890 ml) removed. (Comparatively little inert protein is removed by this step, the main purpose is to reduce the ethanol concentration.) Solid $(\text{NH}_4)_2\text{SO}_4$ was now added (23 g to each 100 ml) when two layers again separated. The top layer was much the smaller, and dark brown, it was removed in a separating funnel and dialyzed against running tap water overnight, final volume 600 ml, *Kat f* = 20,200.

(Stages 4 and 5 bring about a fourfold purification and decrease the volume to $\frac{1}{15}$, this concentration is important for the subsequent $(\text{NH}_4)_2\text{SO}_4$ fractionation which is less effective if the protein concentration is too low.)

Stage 6 The dialyzed solution from stage 5 was brought to pH 5.6 by the addition of $\frac{1}{10}$ vol of M acetate buffer, and roughly fractionated by adding successive portions of solid $(\text{NH}_4)_2\text{SO}_4$ and centrifuging off the resulting precipitates. Fraction 6a (obtained with 1.96M $(\text{NH}_4)_2\text{SO}_4$) which was greyish white and contained little catalase, was rejected.

Fractions 6b, c and d, taken off at 2.27, 2.38 and 2.52M $(\text{NH}_4)_2\text{SO}_4$, were brown and contained most of the catalase, little being left in the final supernatant. These three fractions were combined and dissolved in M/20 acetate pH 5.6 to give a volume of 61 ml, *Kat f* = 40,000.

Stage 7 The combined fractions b, c and d were now carefully fractionated by dropwise addition of a 4M $(\text{NH}_4)_2\text{SO}_4$ solution adjusted to pH 5.6 with NH_4OH . (It was found impossible to standardize these fractionations completely. The exact $(\text{NH}_4)_2\text{SO}_4$ concentration at which catalase begins to precipitate is markedly affected by the catalase concentration, the temperature and probably other factors. It is necessary to proceed empirically, adding the $(\text{NH}_4)_2\text{SO}_4$ drop by drop and with good stirring, taking off fractions at intervals. Fortunately, the colour of the catalase makes it easy to determine its distribution in the precipitates.) Three successive precipitates were taken off, after which little catalase remained in the supernatant fluid. The smaller 1st and 3rd precipitates were visibly paler than the 2nd, and obviously contained colourless protein impurities. The 2nd dark brown precipitate contained the bulk of the catalase and was dissolved in M/20 acetate pH 5.6 to give a volume of 63 ml, *Kat f* = 62,900.

Stage 8 (crystallization) The solution resulting from the last stage was crystallized in three different ways. (i) A portion (10 ml) of the solution was dialyzed against repeated changes of distilled water at 0°. After 5 days (longer than this may be necessary) it had mostly crystallized. (ii) Another 10 ml portion of the solution was cautiously treated with 4M $(\text{NH}_4)_2\text{SO}_4$ until the faintest turbidity appeared. The solution was allowed to stand at room temperature, and by the next day it had almost completely crystallized, leaving a nearly colourless mother liquor. (iii) The rest of the solution (43 ml) was treated with just enough 4M $(\text{NH}_4)_2\text{SO}_4$ to precipitate all the catalase. The precipitate was centrifuged down and redissolved by adding distilled water drop by drop, very slowly and with good stirring, until all but a small portion had dissolved, this was centrifuged off. The supernatant fluid was then saturated with amorphous catalase, it was allowed to stand at room temperature, and most had crystallized within 24 hr. (iv) A smaller second crop of crystals (ivb) was obtained by adding a few drops of $(\text{NH}_4)_2\text{SO}_4$ to the mother liquor of the 1st crop. The four batches of crystals were separately dissolved in M/15 phosphate buffer pH 6.8, well dialyzed, and tested, *Kat f* = 90,000–98,000, which is the highest value we have obtained. There is thus a considerable increase in purity on crystallization.

The yields and purities at each stage of the isolation procedure are shown in Table 1.

Table 1 *Purification of bacterial catalase*

Stage	Volume (ml)	Total protein (g)	Total catalase (g)	<i>Kat f</i>
1 Lyzed bacteria	5,085	203	1.94	910
2 Ethanol supernatant	8,180	40.2	1.69	4,020
3 Chloroform supernatant	7,610	29.5	1.60	5,200
4 First ethanol $(\text{NH}_4)_2\text{SO}_4$ partition	2,760	8.9	1.46	15,650
5 Second ethanol $(\text{NH}_4)_2\text{SO}_4$ partition	600	5.4	1.14	20,200
6 First $(\text{NH}_4)_2\text{SO}_4$ fractionation	61	2.1	0.90	40,000
7 Second $(\text{NH}_4)_2\text{SO}_4$ fractionation	63	0.735	0.485	62,900
8. Crystals (i)	—	0.050	0.050	93,300
(ii)	—	0.065	0.065	98,000
(iii a)	—	0.206	0.206	99,000
(iii b)	—	0.055	0.055	90,000

Properties of the crystalline enzyme

General properties Solutions of the pure enzyme have a red brown colour resembling that of methaemoglobin. Strong solutions (1% or over) are stable for many weeks at 0°, or even at room temperature, if bacterial contamination is avoided, but very dilute solutions such as are used in *Kat f* determinations lose their activity fairly rapidly, and must be tested immediately. In this they resemble liver catalase (Sumner & Dounce, 1937).

Crystalline form The pure enzyme crystallizes as regular octahedra, which are isotropic when viewed between crossed polaroids, they have the same form, however the enzyme is crystallized (Pl 3). In this respect bacterial catalase differs from beef-liver catalase, which according to Sumner & Dounce (1937) may crystallize as needles, plates or prisms, according to the conditions of crystallization.

Prosthetic group Bacterial catalase has a higher activity (*Kat f*) than catalases from other sources (Tables 1 and 3). It is important to discover whether this is to be attributed to differences in the protein

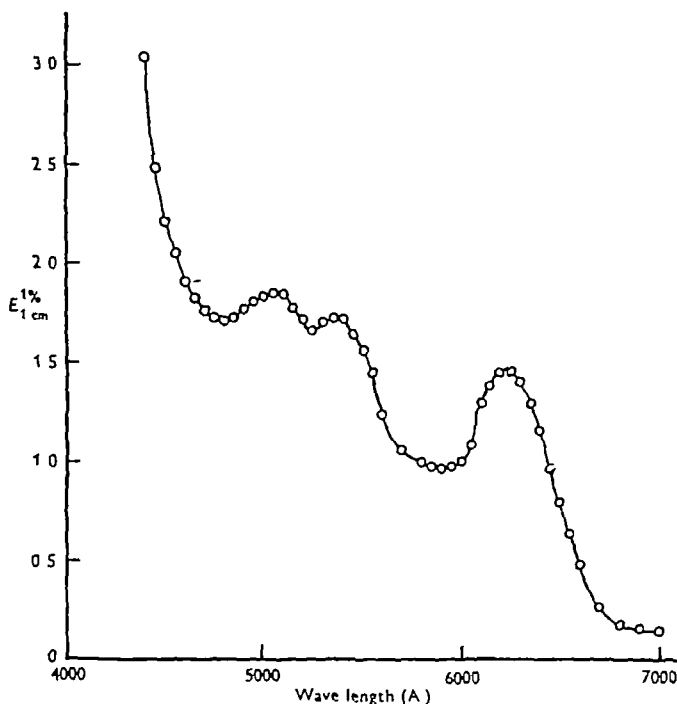


Fig 1 Absorption spectrum of bacterial catalase Crystalline enzyme (*Kat f* = 99,000) in M/15 phosphate pH 6.8

Bacterial catalase is fairly stable at high pH values (e.g. in 0.1N ammonia) but much less stable to acid pH. Horse liver and erythrocyte catalase are stable down to pH 3.2 (Agner & Theorell, 1946), but bacterial catalase is instantly denatured and precipitated at pH 4.0, and fairly rapidly at pH 4.6. Hence, treatment with acetate buffer pH 3.8, which Bonnichsen (1947) used effectively for purifying mammalian catalases, cannot be employed for purifying the bacterial enzyme. Dioxan, which Sumner & Dounce (1937, 1939) used to purify liver catalase, denatures bacterial catalase very rapidly at room temperature and still rapidly at 0°, we were able to obtain fairly pure preparations by Sumner's method working at 0°, but only in very low yields

or the prosthetic group, and the nature of the latter was investigated in some detail.

Strong solutions of bacterial catalase have a characteristic absorption spectrum (Fig 1), showing three bands centred at 506, 545 and 631 mμ (in M/15 phosphate pH 6.8), which are close to the values reported for other catalases. The exact positions and intensities of the bands are affected both by the pH and the nature of the buffer anion (cf Agner, 1942). These data are not always recorded in the literature, it is uncertain, therefore, whether the slight spectroscopic differences observed between different catalases are apparent or real.

On treatment with pyridine and sodium hyposulphite ($\text{Na}_2\text{S}_2\text{O}_4$) in 0.1N NaOH bacterial catalase

gives a typical haemochromogen spectrum, and it forms characteristic compounds with cyanide and azide. Neither azide catalase nor cyanide catalase, nor catalase itself, is reduced by hyposulphite. Azide catalase forms a characteristic pink compound with hydrogen peroxide, which gradually reverts to the original spectrum as the hydrogen peroxide decomposes. This behaviour is exactly similar to that of liver catalase as described by Keilin & Hartree (1937), and the absorption bands of the compounds have similar positions (Table 2)

at 502, 547 and 644 $m\mu$, identical with those of haemin in this solvent. It contained no detectable trace of the 'blue pigment' (biliverdin) that is produced when liver catalases are treated in a similar way (Sumner & Dounce, 1939). On evaporating off the acetone *in vacuo*, the haemin precipitated, leaving a colourless supernatant fluid. The haemin was centrifuged off, washed with water, and dissolved in 10 ml of dilute Na_2CO_3 . Part of this alkaline haematin solution was reserved for conversion to the cyanide and pyridine haemochromogen derivatives, the remainder was immediately coupled with a c 1.5% solution of globin prepared from human blood haemoglobin by the method of

Table 2 *Absorption bands of catalase and catalase haematin derivatives*

(Positions of the absorption bands, determined with the Hartridge reversion spectroscope, are given in $m\mu$)

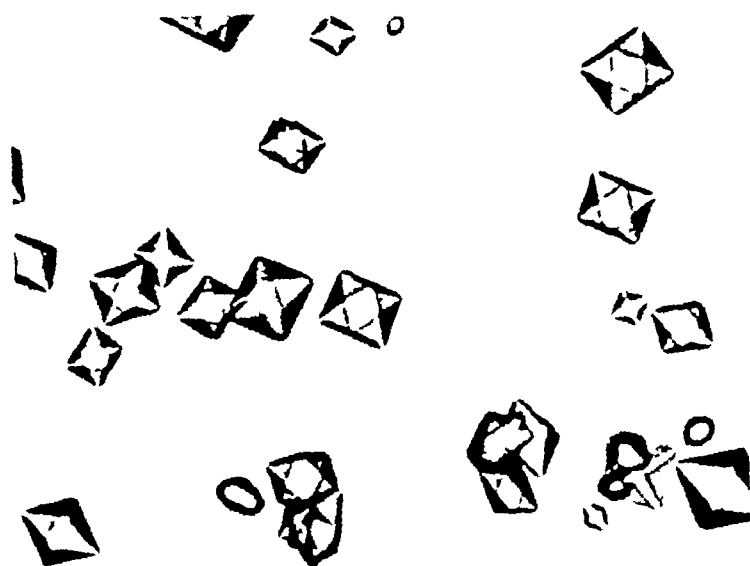
	Catalase derivatives	
	<i>M. lysodeikticus</i> catalase (this paper)	Horse liver catalase (Keilin & Hartree, 1937)
Catalase	506, 545, 631.5	506.5, 544, 629.5
Azide catalase	504, 541, 623	506.5, 544, 624.5
Azide catalase— H_2O_2	548, 588	547, 588
Cyanide catalase	555.5, 592	556.5, 595.5
	Haematin derivatives	
	Bacterial catalase haematin	Blood haematin
Haematin (in ether acetic acid)	505, 545, (584), 638	506, 544.5, (585), 637
Cyanhaematin	547	547
Reduced cyanhaematin	537, 578.5	536.5, 578
Pyridine haemochromogen	525, 557	526, 557
	'Resynthesized haemoglobin' derivatives	
	From globin + bacterial catalase haematin	From globin + blood haematin
Methaemoglobin (alkaline)	542, 575.5, 603.5	541.5, 575.5, 603
Haemoglobin	557	560
Oxyhaemoglobin	540, 577.5	540.5, 577
CO haemoglobin	536.5, 572	537, 572

The above facts strongly suggest that the prosthetic group of bacterial catalase is haematin, but further evidence is needed. This was obtained by splitting off the prosthetic group with acetone hydrochloric acid and coupling the isolated pigment to globin, when it formed methaemoglobin which could be converted to reduced haemoglobin, oxyhaemoglobin and carboxyhaemoglobin, identical with the corresponding derivatives formed from globin and pure haemin. This is proof that the prosthetic group of bacterial catalase actually is haematin, the same experiment also showed that it contains no verdohaematin or similar substance giving rise to biliverdin on treatment with acetone-hydrochloric acid. Details are given below.

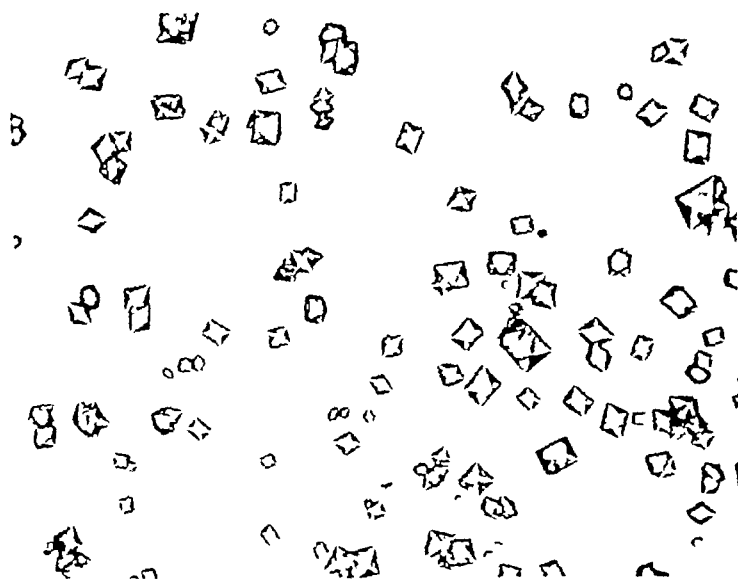
Bacterial catalase (15 ml. of 1.5%) was run into 300 ml of pure acetone containing 5 ml of conc. HCl. The flocculent protein precipitate was filtered off and washed with acetone HCl, the washings being added to the original filtrate. The washed protein precipitate was pure white. The acetone hydrochloric acid filtrate was brown, showing diffuse bands

Anson & Mirsky (1929-30). The globin solution (which was free from denatured globin) was added drop by drop to the solution of catalase haematin until visual and spectroscopic observation showed that all of the latter had coupled with the globin, the solution then showed the absorption bands of methaemoglobin. This was reduced with the minimum quantity of Stokes's reagent (1% FeSO_4 in 2% tartaric acid) required to convert it to reduced haemoglobin. On shaking vigorously with air this was converted to oxyhaemoglobin, which was further converted to CO haemoglobin by saturation with CO. The same series of compounds was formed from the same globin solution by coupling with a freshly prepared solution of recrystallized blood haemin, the absorption bands of both series, and of the other catalase haematin derivatives, are shown in Table 2. The spectra of the two series of haemoglobin derivatives were identical within the errors of reading (Table 2).

For comparison, samples of purified horse liver catalase and crystalline human blood catalase were treated with acetone hydrochloric acid in the same way. The blood catalase behaved exactly like the bacterial catalase, giving a brown acetone solution



(a) Crystallized from $(\text{NH}_4)_2\text{SO}_4$, $\times 500$



(b) Crystallized by dialysis, $\times 200$

Crystalline catalase from *Micrococcus lysodeikticus*

containing, as far as could be ascertained, only haematin. The liver catalase, however, gave a bright blue acetone solution, containing both haematin and biliverdin, as Sumner & Dounce (1939) have described. This could be detected with even a few drops of a 1.5% liver catalase solution, bacterial catalase contains none of the substance (probably verdohaematin, see Lemberg & Legge, 1943) that gives biliverdin on treatment with acetone hydrochloric acid.

Solutions of bacterial catalase and blood catalase are identical in colour to the naked eye, while horse-liver catalase is distinctly greener in colour, and can be seen spectroscopically to have a higher end-absorption in the red. This difference in colour is almost certainly due to the presence of verdohaematin in the liver catalase, and its absence in the other two catalases.

Catalytic activity, haematin content and molecular weight

Table 3 records the *Kat f* and haematin content of crystalline bacterial catalase preparations compared with those of other catalases, and also the ratio *Kat f*/percentage of haematin. Evidently this ratio should be independent of the purity of the catalase preparation provided that impurities present (a) contain no haematin, (b) have no effect on the activity of the enzyme. Fig. 2 shows *Kat f* plotted against

the percentage of haematin for bacterial catalase preparations of varying degrees of purity, the lowest

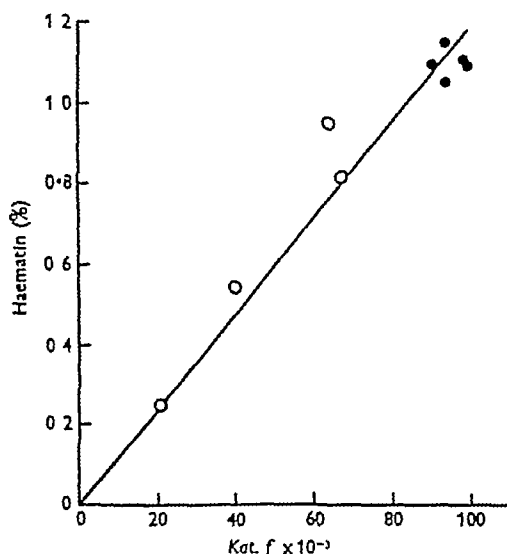


Fig. 2. Relation between haematin content and *Kat f* for bacterial catalase at different stages of purification. Crystalline preparations, ●, amorphous, ○.

only 20% pure. The points are well fitted (within the limits of error) by a straight line passing through the

Table 3. *Catalytic activity and haematin content of bacterial catalase compared with liver and erythrocyte catalases*

Source	<i>Kat f</i>	Haematin (%)	Bile pigment (%)	<i>Kat f</i> /% of haematin	Sedimentation constant	References
Liver catalases						
Ox	30,000–35,000	0.46–0.54	0.49–0.55*	57,000–67,000	11.2×10^{-13}	Sumner & Dounce (1939), Sumner & Gralén (1938)
Horse	25,000–40,000	0.41–0.68	0.37–0.62*	58,000–61,000	—	Sumner, Dounce & Frampton (1940)
	34,000–48,000	0.67–0.92	+	50,000–53,000	—	Keilin & Hartree (1945a)
	50,000	0.89–0.91	+	55,000–56,000	11.3×10^{-13}	Bonnichsen (1947), Agner (1938)
Erythrocyte catalases						
Ox	48,000	?	Nil	?	—	Laskowski & Sumner (1941)
Horse	65,000–70,000	1.042	Nil	62,000–67,000	—	Agner & Theorell (1946)
	65,000	1.08	Nil	60,000	—	Bonnichsen (1947)
Human	50,000	0.83	Nil	60,300	—	Bonnichsen (1947)
	63,000	1.15	Nil	54,700	11.26×10^{-13}	Herbert & Pinsent (1948), Cecil & Ogston (1948)
Bacterial catalase						
<i>M. lysodeikticus</i>	93,300†	1.042	Nil	89,500	—	This paper
	98,000‡	1.103	Nil	89,000	—	This paper
	99,000§	1.096	Nil	90,000	11.0×10^{-13}	This paper, Cecil & Ogston (1948)
	90,000	1.096	Nil	82,200	—	This paper

* From 'bile pigment Fe' analyses

† Crystallized by dialysis, stage 8 (i)

‡ Crystallized by $(\text{NH}_4)_2\text{SO}_4$, stage 8 (ii)

§ Crystallized by $(\text{NH}_4)_2\text{SO}_4$, stage 8 (iii)

|| Crystallized by $(\text{NH}_4)_2\text{SO}_4$, stage 8 (iv)

origin. This shows that our preparations are not contaminated with other iron porphyrin proteins.

Table 3 shows that the haematin content of our preparations is the same as those recorded by other workers for blood catalases and higher than those reported for liver catalases, the *Kat f*, however, is *c* 50% higher than the most active catalases of animal tissues, the average value being *Kat f* = 95,000. Similarly, the ratio *Kat f*/percentage of haematin is higher, being *c* 88,000. To make certain that this is not due to any error in our analytical methods, we prepared crystalline blood catalase by a new method (Herbert & Pinsent, 1948),

Cecil & Ogston (1948) have examined our crystalline preparations in the ultracentrifuge and found them to consist essentially of a single homogeneous protein with sedimentation constant 11.0×10^{-13} (Table 3). In the best preparation examined in the ultracentrifuge, the catalase accounted for 85% of the sedimenting material. As the error of this determination is *c* 5%, we may conclude that our preparations were essentially homogeneous. The haematin content and the agreement between the chemical and ultracentrifugal calculations of the molecular weight lead us to suspect that the small amount of impurity apparently present is probably denatured catalase. If this is so, then the activity

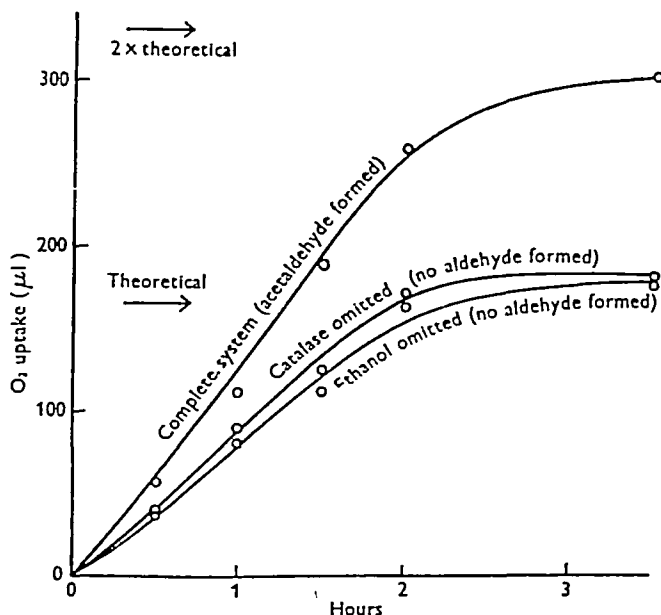


Fig. 3. Coupled oxidation of ethanol. Warburg manometers contained xanthine oxidase 8 mg, hypoxanthine 1 mg, ethanol 0.1 ml, crystalline bacterial catalase 0.8 mg, $M/20$ phosphate pH 7.2, total vol. 3 ml. Temp. 30° , gas phase air.

and determined its activity and haematin content in parallel with determinations on bacterial catalase. Any systematic errors in our *Kat f* and haematin determinations would apply equally to both enzymes. In fact our values for blood catalase (Table 3) are in good agreement with those of other workers. We concluded, therefore, that bacterial catalase actually has a considerably higher activity, whether measured on a dry weight or on a haematin basis, than the catalases of mammalian tissues. Since the prosthetic groups are the same, the difference must be attributed to the protein component of the enzyme.

Assuming our crystalline preparations to be pure, the mean haematin content of 1.09% corresponds to a molecular weight of $58,000 \times n$, where n is the number of haematin groups/mol. Assuming $n = 4$, as for catalases of mammalian tissues, this gives a value for the molecular weight of 232,000.

of the completely pure enzyme may be some 10% higher than the values given above.

Once the molecular weight and *Kat f* of the pure enzyme are known, it is possible to calculate the 'Turnover Number', defined by Warburg & Christian (1933) as the number of molecules of substrate decomposed by one molecule in 1 min. The relation between Turnover no. and *Kat f* is obtained as follows. The initial velocity is obtained from the velocity constant k_0 by the first order reaction equation $\left(\frac{-dS}{dt}\right)_{t=0} = 2.303 k_0 S_0$. By definition,

$$Kat f = \frac{k_0}{g \text{ catalase}/50 \text{ ml}} = 20 k_0 / EM, \text{ where } S_0 \text{ and } S \text{ are the concentrations of hydrogen peroxide at } 0 \text{ and } t \text{ min, and } E \text{ is the concentration of catalase (all in mol/l), and } M \text{ is the molecular weight of catalase}$$

Hence,

$$\text{Turnover no} = \frac{1}{E} \left(\frac{-dS}{dt} \right)_{t=0} = Kat f \times 0.115 \bar{M} S_0$$

For bacterial catalase of mol wt = 232,000 and $Kat f = 95,000$, under the test conditions (0.0075 M- H_2O_2 at 0°), this gives a Turnover no of 19×10^6 , the highest recorded for any enzyme

By similar calculations, the relation between $Kat f$ and the Q_{O_2} ($\mu l O_2$ evolved/mg of enzyme/hr) is given by

$$Q_{O_2} = Kat f \times 77,500 S_0$$

Under the test conditions, bacterial catalase has a Q_{O_2} of 55×10^6 (The activity of catalase is usually measured at low hydrogen peroxide concentrations, when the percentage of total enzyme combined with substrate is small and proportional to the substrate concentration, so that the reaction is first order. Under these conditions k and $Kat f$ are independent of the initial substrate concentration, but the Turnover no and Q_{O_2} are directly proportional to the hydrogen peroxide concentration, which should always be stated.)

Coupled oxidation of alcohols

Keilin & Hartree (1936, 1945b) have shown that if catalase and ethanol are added to any enzyme system which produces hydrogen peroxide, the latter is used by the catalase to bring about a 'coupled oxidation' of the ethanol to acetaldehyde. In other words, under these special conditions (continuous slow supply of hydrogen peroxide at a very low concentration), the catalase acts as a peroxidase.

Fig. 3 shows that bacterial catalase behaves exactly like liver catalase in this respect. Xanthine oxidase hypoxanthine was used as the source of hydrogen peroxide, the coupled oxidation is shown by a doubling of the theoretical oxygen uptake and the formation of acetaldehyde, recognized by its smell and the brown coloration imparted to the potassium hydroxide papers in the manometer cups. Acetaldehyde is only formed when catalase, ethanol, xanthine oxidase and hypoxanthine are all present.

DISCUSSION

The objects of this work were twofold: to investigate the use of lysozyme for the liberation of enzymes from the bacterial cell, and to apply this technique to the isolation of bacterial catalase.

Our present results indicate that the lysozyme technique is an excellent one, though not entirely without drawbacks. It is, of course, only applicable to lysozyme sensitive micro organisms. The viscous substances released from the bacteria by lysozyme (or possibly formed by its action) are a decided hindrance in the initial stages of enzyme purification. Nevertheless, the simplicity of the technique, the

fact that it is unlikely to destroy labile enzymes, and its ready applicability on either a large or a small scale, should make it a useful tool in studying the intracellular components of bacteria.

The isolation of the catalase of *M. lysodeikticus* was undertaken partly in the hope that interesting differences might be revealed between the bacterial enzyme and the catalases of mammalian tissues. In fact, the differences observed are less striking than the similarities. The prosthetic group is the same as that of mammalian catalases, the molecular weight is the same, and there are the same number of haematin groups in the molecule. The main difference is that the catalytic activity of the bacterial enzyme is considerably higher than that of mammalian catalases, this difference must be attributed to the protein component of the enzyme.

The protein components of sheep, ox, horse and human catalases have all been shown to be different by immunological methods (Tria, 1939; Campbell & Fourt, 1939; Bonnicksen, 1947), and there is little doubt that the protein of bacterial catalase is different again. All these proteins, however, when combined with haematin, have the common property of catalyzing specifically the decomposition of hydrogen peroxide. It seems reasonable to suppose that this common property is related to some common structural element of the molecule—possibly some particular grouping of certain amino-acids—on which the catalytic activity depends. A careful study of the same enzyme isolated from several different sources might throw considerable light on the nature of enzymic catalysis. Catalase, which has now been isolated pure from so many different sources, should be a suitable enzyme to choose for such an investigation.

Another point of interest arising from this work is the remarkably high concentration of catalase found in *M. lysodeikticus*. This can be calculated from the $Kat f$ values of the pure enzyme (95,000) and the lysed bacteria (800–1800 in different batches), giving a catalase content for this organism of 1–2% of its dry weight. This is about ten times the catalase content of human red blood corpuscles (Herbert & Pinsent, 1948). It can also be calculated that a single bacterial cell (dry weight taken as 2.5×10^{-13} g) contains $10\text{--}20 \times 10^3$ enzyme molecules.

Such calculations raise the question of the function of catalase in *M. lysodeikticus*. The above concentrations of catalase would enable each bacterial cell to decompose c. 35–70 times its own weight of hydrogen peroxide/min (at 0° and hydrogen peroxide concentration of 0.01M). Such large amounts of peroxide could scarcely be formed as a result of cell metabolism, and it is difficult to devise any teleological explanation for this high enzyme concentration unless, as the discovery of 'coupled

oxidation' by Keilm & Hartree (1936, 1945*b*) suggests, catalase has other functions besides the simple decomposition of hydrogen peroxide

SUMMARY

1 The use of lysozyme in the study of intracellular bacterial enzymes and some phenomena noticed when lysozyme acts on concentrated bacterial suspensions are discussed

2 Using lysozyme to liberate the enzyme from the bacterial cell, catalase has been isolated from *Micrococcus lysodeikticus* in crystalline form

3 Bacterial catalase is in many respects similar to the catalases of animal tissues. The crystalline enzyme contains c. 1.1% of haematin, identical with ordinary blood haematin

4 It differs from liver catalases, but resembles erythrocyte catalases, in containing no verdo haematin

5 Haematin content and ultracentrifugal data indicate a molecular weight of c. 230,000, with four haematin groups/molecule

6 The catalytic activity is considerably higher than that of mammalian tissue catalases, the difference is to be attributed to the protein component of the enzyme

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Crystalline Human Erythrocyte Catalase

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The enzyme catalase has been isolated in crystalline form from ox liver (Sumner & Dounce, 1937), horse liver (Agner, 1942), lamb liver (Dounce, 1942), ox erythrocytes (Laskowski & Sumner, 1941), human and horse erythrocytes (Bonnichsen, 1947). In the preceding paper (Herbert & Pinsent, 1948) we describe the isolation of crystalline catalase from a bacterium, *Micrococcus lysodeikticus*.

With the exception of human blood catalase, all these enzymes have been reported to contain about 0.097% Fe or 1.1% Fe porphyrin, corresponding to four iron porphyrin groups in a molecule of molecular weight 230,000, in bacterial and erythrocyte catalases. All four iron porphyrin groups are haematin, while in liver catalases a varying proportion may be verdohaematin or a related compound. This similarity between the catalases of three mammalian and one bacterial species is remarkable, and the reported exception of human blood catalase is unexpected. This enzyme was crystallized by Bonnichsen (1947), who found it to contain only 0.077% Fe and 0.83% haematin, and stated 'if we assume the same molecular weight as for the other catalases, this would mean that this catalase contains only three haematin groups'.

We have isolated crystalline catalase from human red cells by a new method. Contrary to Bonnichsen's findings, it contained 1.15% haematin, corresponding to four haematin groups/molecule, if the molecular weight is assumed to be 220,000. Since no ultracentrifugal measurements have hitherto been reported for erythrocyte catalases, our enzyme was submitted to Dr R. Cecil, who found it to have a sedimentation constant of 11.26×10^{-13} (Cecil & Ogston, 1948), identical with the recorded values for liver catalases. Our preparation had a *Kat f* of 63,000 and a ratio *Kat f*/percentage of haematin of 54,400, which are similar to the values reported for other mammalian catalases (see Table 2, Herbert & Pinsent, 1948).

These results show human erythrocyte catalase to be essentially similar in catalytic activity and molecular weight to all other erythrocyte catalases, and to have the same number (four) of haematin groups/molecule. As our method of isolation evidently produces a purer enzyme than that of Bonnichsen (1947), and also gives higher yields, the details were thought to be worth placing on record.

EXPERIMENTAL

Methods. All methods used for determination of *Kat f*, haematin content, etc., were exactly as described by Herbert & Pinsent (1948).

Isolation of crystalline human erythrocyte catalase

No particularly novel methods have been used in our isolation procedure. The first step consists in shaking the haemolyzed red cells with ethanol and chloroform (cf. Tsuchihashi, 1923), this removes all the haemoglobin, leaving the catalase in the supernatant fluid. The catalase is then absorbed on calcium phosphate gel. It may be eluted from the gel with 0.05N ammonium hydroxide or disodium phosphate, but several washes with rather large volumes are necessary for complete elution, so that the final eluate is very dilute. Instead, we decompose the gel by shaking with potassium oxalate and centrifuging off the precipitated calcium oxalate. This gives quantitative recoveries in a small volume.

These two steps effect a hundredfold purification and give an enzyme approximately 20% pure, from which pure, crystalline catalase may easily be obtained by careful fractionation with ammonium sulphate. Controlled salt fractionation has not much been used by previous workers on catalase, who have mostly preferred fractionation with organic solvents, our experience shows it to be as efficient as well as a simple technique.

The following are the details of a typical preparation.

Stage 1. The red cells were removed from citrated human blood by centrifuging, and washed twice on the centrifuge with 0.9% NaCl. 6.84 l of packed red cells were laked with an equal volume of distilled water, giving a solution containing 1690 g total protein and 2.71 g pure catalase, *Kat f* = 101 (Note 1).

Stage 2. The laked red cells were treated with 0.44 vol of a 3:1 ethanol:chloroform mixture, and stirred vigorously for 15 min. The sticky mass of precipitated haemoglobin was removed by straining through muslin and well pressed out. The rather turbid solution was filtered through a pad of 'Hyflo Super Cel,' giving 14.6 l of a clear, pale yellow filtrate showing no haemoglobin spectrum. *Kat f* = 1020, yield 70%.

Stage 3. The above filtrate was treated (Note 2) with 2 l of a 1.5% suspension of $\text{Ca}_3(\text{PO}_4)_2$ gel (Keilin & Hartree, 1938) and stood 1 hr to allow the gel to settle. Most of the supernatant fluid could then be decanted off and the re-

maimder separated by centrifuging. The gel had adsorbed a considerable quantity of protein but very little catalase. A second addition of 30 g of $\text{Ca}_3(\text{PO}_4)_2$ (Note 2) adsorbed almost all the catalase, and was collected by decantation followed by centrifuging. The packed $\text{Ca}_3(\text{PO}_4)_2$ precipitate was shaken vigorously with 600 ml of 0.5M potassium oxalate in 0.5M NaH_2PO_4 (Note 3). The calcium oxalate was centrifuged off and washed twice with water, the washings being added to the first eluate. This gave 1620 ml of a brown solution with a clearly visible catalase spectrum, $Kat f = 10,400$, overall yield 56%.

Stage 4 Solid $(\text{NH}_4)_2\text{SO}_4$ was added to the above solution (30 g/100 ml.), and the greyish brown precipitate collected by centrifuging and dissolved in 100 ml water, 20 ml of π acetate buffer pH 4.0 were then added and the solution allowed to stand 2 hr (cf Bonnichsen, 1947). A flocculent greyish precipitate formed and was removed by centrifuging, leaving a clear greenish brown liquor which was adjusted to about pH 5.6 with Na_2HPO_4 , $Kat f = 21,800$, overall yield 36%.

Stage 5 The catalase solution from stage 4 was treated with 4M $(\text{NH}_4)_2\text{SO}_4$, added drop by drop with good stirring. A small, greyish brown precipitate which appeared at an $(\text{NH}_4)_2\text{SO}_4$ conc of 1.4M (Note 4) was removed by centrifuging. Careful addition of $(\text{NH}_4)_2\text{SO}_4$ was continued until a faint turbidity appeared, on standing at room temperature this rapidly increased and developed a silken sheen, noticeable on stirring, like a suspension of bacteria. Microscopic examination showed it to consist of very fine needle shaped crystals. After standing 1 hr these were centrifuged down, and a further crop of crystals obtained from the mother liquor by adding a little more $(\text{NH}_4)_2\text{SO}_4$. The two crops of crystals were pooled and dissolved in 50 ml of water, total 710 mg or 26% overall yield on the red cell haemolysate. The $Kat f$ was 50,200 and the haematin content 1.05%. After two recrystallizations by the same method, the $Kat f$ was raised to 63,000 and the haematin content to 1.15%.

Note 1 This batch of red cells had been kept for 13 days at 2° before using. In another preparation where the blood was used fresh, the washed, laked red cells had a $Kat f$ of 106. Both batches were pools from several donors.

Note 2 The quantity of calcium phosphate required varies from one preparation to another, and should be determined by a small scale trial, treating, e.g. 10 ml samples of enzyme with 1, 2, 3 and 4 ml of gel suspension, centrifuging, and estimating the catalase left in the supernatant fluid.

Note 3 When $\text{Ca}_3(\text{PO}_4)_2$ is decomposed with oxalate the solution becomes strongly alkaline through the formation of K_3PO_4 , hence NaH_2PO_4 is added to keep the pH neutral.

Note 4 The exact $(\text{NH}_4)_2\text{SO}_4$ concentration cannot be standardized, as it depends on many factors, including the catalase concentration, it is necessary to proceed empirically (cf Herbert & Pinsent, 1948).

Properties of the crystalline enzyme

As already mentioned, the enzyme crystallizes from $(\text{NH}_4)_2\text{SO}_4$ in fine needles. Their refractive index is very similar to that of the mother liquor, which makes them difficult to photograph. On dialysis for many weeks against distilled water the enzyme crystallized in large irregular plates. It is more difficult to crystallize in this way than bacterial catalase, electrodialysis would probably be more

efficient (cf Bonnichsen, 1947). Solutions of the crystals in 0.05M phosphate pH 6.8 are reddish brown, and show absorption bands at 630, 544 and 505 m μ (Hartridge reversion spectroscope). Treatment with pyridine, sodium hydroxide and sodium hyposulphite produces the characteristic spectrum of pyridine haemochromogen. On treating with acetone hydrochloric acid (cf Herbert & Pinsent, 1948) the prosthetic group is split off, and appears to consist entirely of haematin, no trace of biliverdin could be detected.

The purest sample obtained had a $Kat f$ of 63,000, and contained 1.15% haematin. If this is the pure enzyme, the molecular weight is $n \times 55,000$, where n is the number of haematin groups/molecule, for four haematin groups, the molecular weight would be 220,000. This sample was examined in the ultracentrifuge by Cecil & Ogston (1948) who found it to consist in the main of a homogeneous protein with a sedimentation constant of 11.26×10^{-13} . This is in good agreement with the values of 11.2×10^{-13} for beef liver catalase (Sumner & Gralén, 1938) and 11.2×10^{-13} for horse liver catalase (Agner, 1938). This component accounted for 72% of the total refractive increment, and traces of another coloured protein were present in the ultracentrifuge photographs. Unfortunately some time had elapsed between the preparation of this sample and its examination in the ultracentrifuge, during which period the activity had decreased from the original value of $Kat f = 63,000$ to 44,300, a decrease of 29%. We suspect, therefore, that the coloured impurity present was denatured catalase.

A rough estimate of the minimum molecular weight from the sedimentation constant is 176,000, assuming a spherical unhydrated molecule. If the molecule is not spherical, which is certainly the case (Sumner & Frampton, 1940), the molecular weight would be higher. It was unfortunately impracticable to perform measurements of partial specific volume and diffusion constant.

DISCUSSION

The results show that (within the limits of error of the various methods) human blood catalase has essentially the same $Kat f$, haematin content and molecular weight as other red cell catalases, and like them, has four haematin groups/molecule. The reason for the discrepancy between our results and those of Bonnichsen (1947) is not clear. His preparations, however, had about the same $Kat f$ /percentage of haematin ratio as ours, although the absolute values of both were lower. The simplest explanation would be that his preparations were not pure, we found several recrystallizations necessary to obtain our purest enzyme. Our method of purification is simpler than any hitherto described, and should be applicable to the red cells of other species.

Crystalline erythrocyte catalase may now be considered a readily available enzyme

It is interesting to calculate the catalase content of the human erythrocyte. This is obtained from the $Kat f$ of the pure enzyme (63,000) and of washed red cells, which in two different preparations was found to be 101 and 166. Hence catalase accounts for about 0.16–0.26% of the total dry matter of the human red blood corpuscle.

SUMMARY

1 The isolation of crystalline catalase from human red cells is described. The method is simple and the

yield of crystalline enzyme is about 25% of that present in the red cell.

2 The crystalline enzyme had a $Kat f$ of 63,000 and a haematin content of 1.15%. The sedimentation constant was 11.26×10^{-13} . The molecular weight was calculated to be 220,000 from the haematin content. There are four haematin groups/molecule, no verdohaematin is present.

Our thanks are due to Miss M. Mackay of the Medical Research Council's Blood Products Research Group, for kindly supplying us with large amounts of human red cells.

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Examination of Crystalline Catalases in the Ultracentrifuge

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Three samples of crystalline catalase were supplied by Dr Denis Herbert (Herbert & Pinsent, 1948 *a, b*). They were examined in a Svedberg oil turbine ultracentrifuge by the

hydrogen phosphate 0.05M, sodium dihydrogen phosphate 0.05M.

The speed was approximately 1015 rev./sec., and the wave length of light used 620–660 m μ . For the



Fig 1



Fig 2

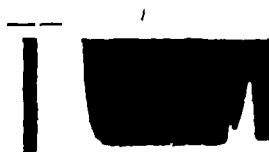


Fig 3a



Fig 3b

Fig 1 Sample AI (bacterial catalase) approx. 25 min. after reaching full speed.

Fig 2 Sample AII (bacterial catalase) approx. 25 min. after reaching full speed.

Fig 3a Erythrocyte catalase immediately on reaching full speed, showing heavier component.

Fig 3b Erythrocyte catalase approx. 25 min. after reaching full speed.

Note: The different appearance of the meniscus in Figs 1 and 2 was due to the use of a layer of paraffin in these two runs.

method of Philpot (1938). The solutions all contained approximately 0.5% catalase in a buffer of composition sodium chloride 0.2M, disodium

calculation of the sedimentation constants, the partial specific volume was taken as 0.73 ml/g (Sumner & Gralén, 1938).

Bacterial catalase (from Micrococcus lysodeikticus)

Sample A I This had an activity corresponding to a *Kat f* of 99,000 (Herbert, private communication) It gave a single homogeneous component with some heavier and lighter impurities (Fig 1) The $S_{20}(\text{corr})$ was 11.0×10^{-13} , and integration of the boundary gave 85% of the initial refracting material

Sample A II This was another preparation of slightly lower activity, *Kat f* = 90,000 (Herbert, private communication) It also gave a single homogeneous component with heavier and lighter impurities (Fig 2), but integration gave only 70% of the initial refracting material The $S_{20}(\text{corr})$ was again 11.0×10^{-13}

Human erythrocyte catalase

A single sample was examined The *Kat f* when first prepared was 63,000, but during keeping it dropped to 44,300 (Herbert, private communication), suggesting that some denaturation had taken place The sedimentation diagram was similar to that of the two previous samples except for a

larger proportion of the heavier impurity, possibly denatured catalase This impurity can be seen clearly in an early photograph (Fig 3a), but it dispersed as the run proceeded (Fig 3b) suggesting heterogeneity Some of the pigment was seen to be associated with it The $S_{20}(\text{corr})$ of the main component was 11.2×10^{-13}

CONCLUSION AND SUMMARY

1 The ultracentrifugal data suggest that, while none of the preparations is fully homogeneous, there is in each case a main component which is associated with most of the pigment

2 The sedimentation constants of this component in preparations from *Micrococcus lysodeikticus* and from human blood are similar (11.0 and 11.2×10^{-13} respectively), though the absolute difference is probably outside the experimental error

3 The value of 11.2×10^{-13} for the sedimentation constant of human blood catalase agrees with Agner's (1938) value of 11.2×10^{-13} for horse liver catalase and with Sumner & Gralen's (1938) value of 11.3×10^{-13} for cow liver catalase

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Studies in Detoxication

17 THE FATE OF CATECHOL IN THE RABBIT AND THE CHARACTERIZATION OF CATECHOL MONOGLUCURONIDE

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The main object of this investigation was to find out if conjugation of catechol occurs *in vivo* on one or both of its hydroxyl groups The study of the fate of catechol was also necessary because it had a bearing on other problems being studied in this laboratory, namely, the metabolism of adrenaline (cf Dodgson, Garton & Williams, 1947) and of benzene (cf Porteous, Smith & Williams, 1947)

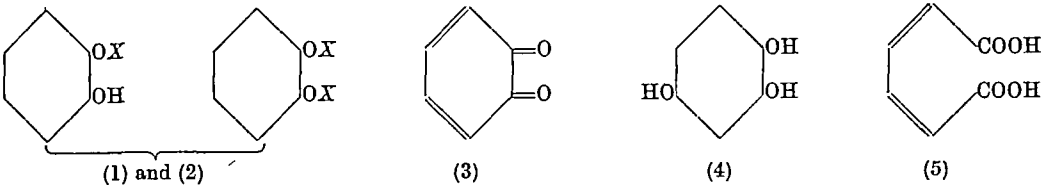
Despite the importance of catechol compounds in biochemistry, no significant study of the fate of catechol in the intact animal has been made other than that of Baumann (1876) who showed that

administration of catechol to dogs caused an increased output of ethereal sulphate However, numerous studies have been carried out *in vitro* of the formation and oxidation of catechol by enzymes of the tyrosinase type (see review by Nelson & Dawson, 1944)

From a consideration of the structure of catechol and of the results of enzyme studies, it is clear that it may form in the body (1) an ethereal mono- or disulphate or both, (2) a mono- or di glucuronide or both, (3) *o* benzoquinone by oxidation, (4) hydroxyquinol (1,2,4 trihydroxybenzene) and its conju-

gates, by oxidation, and (5) muconic acid by ring fission as suggested by Drummond & Finar (1938) These possibilities are formulated as follows

oral dose is in the region of 200 mg /kg (Masing, 1882, Brieger, 1879) In our experiments catechol (British Drug Houses Ltd, m p 103°), dissolved in water, was fed by



where X=an ethereal sulphate or glucuronic acid group

Evidence will be presented to show that the metabolites excreted are mainly the monoglucuronide and monosulphate of catechol together with small amounts of conjugated hydroxyquinol

stomach tube We found that in five rabbits 100 mg /kg had no apparent effect, at 175 mg /kg slight toxic effects were apparent, at 200 mg /kg the rabbits showed immediate signs of distress such as rapid breathing and twitching, with occasional paralysis of the limbs and squeaking After about 3 hr the animals appeared to be normal One experiment was carried out using a dose of 250 mg /kg which was fatal within 24 hr

EXPERIMENTAL

Animals Giant chinchilla rabbits were used and fed on a diet of 50 g Lever's cubes and water daily unless other wise stated.

The effect of catechol on rabbits Catechol is a relatively toxic substance For cats and rats an oral dose of 50 mg /kg is lethal (Dietering, 1938), whereas for rabbits the lethal

Quantitative results

Ethereal sulphate and glucuronic acid outputs Ethereal sulphate excretion was determined by the method of Williams (1938) and glucuronic acid excretion according to Hanson, Mills & Williams (1944) The results are given in Table 1 and Fig 1 illustrates a single experiment

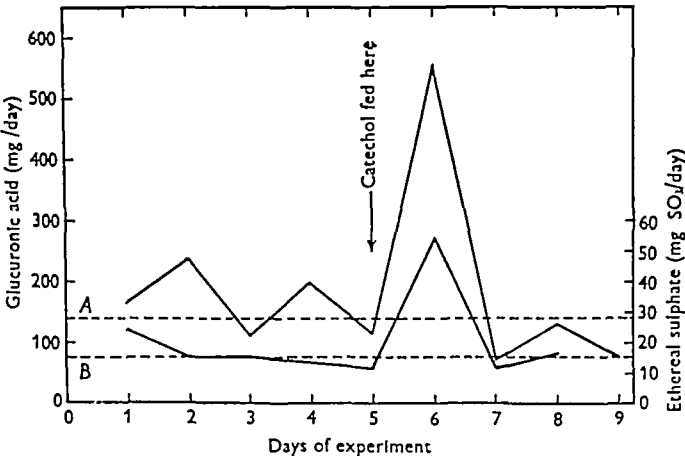


Fig 1 The effect of feeding catechol (300 mg) on the glucuronic acid and ethereal sulphate output of rabbit no 113 (3 kg wt) Upper curve, glucuronic acid lower curve, ethereal sulphate Dotted lines show average normal values for (A) glucuronic acid and (B) ethereal sulphate

Table 1 The glucuronic acid and ethereal sulphate excretion of rabbits receiving catechol orally

Rabbit no	Weight (kg)	Dose (mg)	Normal values of		Extra ethereal sulphate (mg)	Extra glucuronic acid (mg)	% of dose excreted	
			Ethereal sulphate (mg/day)	Glucuronic acid (mg/day)			As sulphate	As glucuronide
97* ♂	2.6	262	28.5	—	31.5	—	16.5	—
98* ♂	3.1	322	28.5	—	54.2	—	18.9	—
113† ♀	3.0	300	15.3	141	38.8	412	17.7	77.9
115† ♀	2.6	260	—	171	—	266	—	60.7
117† ♀	2.7	270	—	117	—	341	—	71.6
Average							17.7	70

* Diet, 50 g Lever's cubes + 100 g cabbage per diem
† Diet 50 g Lever's cubes + water ad lib per diem

Table 1 shows that about 17–18% of the catechol fed is excreted as a sulphate and about 70% as a glucuronide, assuming that monoconjugates are excreted. Furthermore, about 87–88% of the catechol fed can be accounted for by these conjugates excreted within 24 hr of dosing. In the case of rabbit 113, it had been possible to carry out both estimations simultaneously and here the sum of the ethereal sulphate and glucuronide output is 95%. These results indicate that the main bulk of the catechol fed is excreted conjugated. A small amount of free catechol is also excreted which we assess at no more than 5%, for we isolated about 2% of the dose as derivatives of catechol (see below).

The isolation of catechol metabolites

(a) *Catechol urine* Rabbits receiving oral doses of catechol excrete a slightly alkaline urine, which is normal in colour but darkens on standing. It gives the green colour characteristic of free catechol with FeCl_3 , but does not reduce Fehling's or Benedict's reagents. It gives a strongly positive naphthoresorcinol reaction.

(b) *Detection of free catechol* Urine (550 ml in 24 hr) from three animals after the feeding of a total of 0.68 g of catechol was acidified with a few drops of conc. HCl , and extracted with ether for 1 hr in a continuous extractor. The ether was removed at 20° under reduced pressure leaving 300 mg of a brownish gum. This was dissolved in a few ml of 40% (w/v) NaOH and benzoylated in the usual manner. Repeated recrystallization of the product from 95% ethanol yielded 10 mg (0.7% of dose) of catechol dibenzoate, m.p. 83°, or 84.5° mixed with synthetic material (m.p. 85°).

(c) *Isolation and characterization of catechol monoglucuronide* (i) *Preparation of the glucuronide gum* A total of 7.59 g of catechol was fed to 12 rabbits and a 24 hr urine (1750 ml) collected. The urine was clarified by centrifuging and made faintly acid (grey with congo red paper) by adding glacial acetic acid. Saturated lead acetate solution (350 ml) was then added and the precipitate filtered off. The filtrate was made faintly alkaline with a little ammonia (sp. gr. 0.88) and treated with 200 ml saturated basic lead acetate solution. The white precipitate was filtered off, washed well with cold water, made into a thin cream with water and the lead was removed completely with H_2S . The filtrate from the lead sulphide was taken to dryness *in vacuo* at 45–50°, and there remained a brown gum (12.5 g) which showed a very slight reducing action towards Benedict's reagent and contained free catechol as indicated by its reaction with FeCl_3 . The gum was taken up in absolute ethanol and on standing overnight a sludge of inorganic matter separated. This was removed by filtration and then the ethanolic solution was reduced to 30 ml *in vacuo*. This solution was now poured into 300 ml ether. Most of the glucuronide remained in solution but about 2 g of gummy material containing much inorganic matter were thrown out. After removal of this, the ethanol ether solution was concentrated *in vacuo* to a clear brown gum (10 g). At this stage the purified gum gave a strong green colour with FeCl_3 , a strong naphthoresorcinol reaction and reduced Benedict's reagent very slightly. Attempts to crystallize the glucuronide or to prepare crystalline salts with benzylamine, *o* or *p* toluidine or benzidine were unsuccessful.

(ii) *Removal of free catechol from the glucuronide gum* The gum (6 g) was dissolved in 75 ml water and the solution

was extracted with 3 × 100 ml portions of ether. The extract was dried with anhydrous Na_2SO_4 and taken to dryness *in vacuo* at 20°. The semi-crystalline residue (730 mg) gave intense colour reactions for catechol but gave no tests for hydroxyquinol (see below). It was dissolved in 12 ml 2*N* NaOH and immediately mixed with acetone (10 ml) containing *p*-toluenesulphonyl chloride (1 g). The mixture, which turned purple, was shaken vigorously for 15 min and then poured into 30 ml cold water. The white crystalline precipitate (300 mg, m.p. 150°) was collected and recrystallized from absolute ethanol. The product formed colourless needles and was identified as catechol di-*p*-toluenesulphonate, m.p. 161° (Found C, 57.8, H, 4.6, S, 14.95. $\text{C}_{20}\text{H}_{18}\text{O}_6\text{S}_2$ requires C, 57.4, H, 4.3, S, 15.3%). This appears to be a new compound, an authentic specimen, m.p. 162–163°, and 161–162° when mixed with the material described above, has been prepared in another investigation (Porteous & Williams, unpublished). The yield of catechol ester corresponded to 1.8% of the catechol fed.

(iii) *Conversion of the glucuronide gum into the methyl ester of guaiacol triacetylglucuronide* The solution after removal of catechol was taken to dryness and the residual gum (5.2 g) was dissolved in 30 ml absolute ethanol and treated with 250 ml of a saturated ethereal solution of diazomethane. The mixture was kept at room temperature overnight. A slight precipitate of inorganic material was removed by filtration. The solution on evaporation yielded a pale yellow gum. Since the gum was acidic to litmus and incompletely soluble in ether, the treatment with diazomethane was repeated and on removal of the solvents a pale yellow, neutral, ether soluble gum was obtained. This gum, which is presumably guaiacol glucuronide methyl ester, could not be induced to crystallize. It was therefore dissolved in 10 ml pyridine followed by 15 ml acetic anhydride and the mixture kept at room temperature overnight. On pouring into 50 ml ice water a semi-solid gum was precipitated. This gum was now exhaustively extracted with chloroform yielding an orange yellow solution which was washed twice with 2*N* HCl , then twice with 10% (w/v) Na_2CO_3 solution and finally with water. The washed solution was now treated with charcoal, filtered and dried over CaCl_2 . Removal of the solvent on the water bath left a clear yellow gum soluble in ethanol, acetone, ether and benzene but insoluble in water. Crystallization of the gum was achieved by dissolving in a minimum of acetone and then adding it to a large volume of warm water. Any cloudiness was dispersed by addition of a little acetone. After standing several days at room temperature, crystals contaminated with gum gradually appeared. The crystals were again recrystallized from a large volume of acetone water (yield 2.83 g). The compound was now easily recrystallized (charcoal) from aqueous ethanol and formed thick colourless needles, m.p. 107°. It gave the correct analysis for *o*-methoxyphenyltriacetylglucuronide methyl ester (guaiacol triacetylglucuronide methyl ester) (Found C, 54.9, H, 5.5, OMe, 13.0. $\text{C}_{20}\text{H}_{24}\text{O}_{11}$ requires C, 54.5, H, 5.5, OMe, 14.1%). $[\alpha]_D^{18} = -49.3^\circ$ ($c=1$ in ethanol). It gave a positive naphthoresorcinol reaction for glucuronide acid on prolonged boiling (5 min) with the reagents.

(iv) *Acid hydrolysis of the acetylated methyl ester* Isolation of guaiacol The methyl ester (0.75 g) was heated under reflux for 6 hr with 4*N* HCl (50 ml) and ethanol (5 ml). The mixture, which smelt of guaiacol, was cooled and then

extracted with 3×75 ml. portions of ether. The combined ether extracts were extracted with small portions of 10% (w/v) NaOH solution (15 ml. in all). This alkaline extract which now contained the guaiacol was shaken for 10 min with 5 ml acetone containing 0.75 g *p* toluenesulphonyl chloride. The resulting brown solution was poured into 200 ml. water, but no solid appeared. The mixture was therefore heated at 60° for 1 hr when a white precipitate appeared. After cooling, the precipitate (200 mg) was collected and repeatedly recrystallized from ethanol. The product finally had m.p. $81-82^\circ$, and 82° mixed with authentic guaiacol *p* toluenesulphonate, m.p. 82° (Found C, 60.3, H, 5.0, S, 11.65. Calc. for $C_{14}H_{14}O_4S$: C, 60.4, H, 5.1, S, 11.5%). The residual solution, after ether extraction of the guaiacol, gave a positive naphthoresorcinol reaction and after neutralization reduced Fehling's and Benedict's reagents, thus showing the presence of glucuronic acid.

(d) *Detection of hydroxyquinol (1,2,4 trihydroxybenzene) in catechol urine*. Other work in this laboratory (Porteous & Williams, unpublished) had shown that benzene is converted in the rabbit to hydroxyquinol and we believe that this phenol is formed from benzene via catechol. It seemed likely therefore that on feeding catechol itself some hydroxyquinol might be produced.

Hydroxyquinol can be readily detected in the presence of catechol by two colour reactions

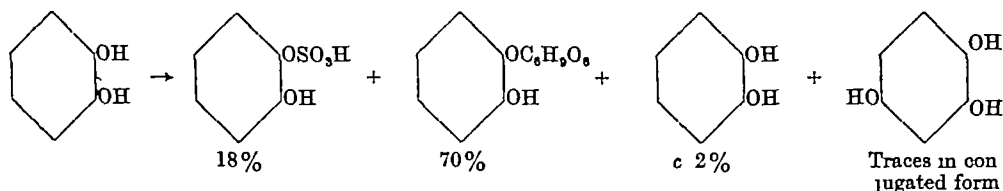
(i) *The sulphuric acid test*. If small amounts of hydroxyquinol are gently warmed with conc. H_2SO_4 , an intense red colour develops immediately. Phenol, catechol, quinol, pyrogallol and phloroglucinol do not give colours with H_2SO_4 under these conditions, whilst resorcinol gives a faint orange colour. By this test small amounts of hydroxy

peroxide free ether. Catechol and hydroxyquinol were detected in the extract up to 24 hr after beginning the extraction. After 27 hr extraction neither catechol nor hydroxyquinol was detected. The combined extracts were evaporated to dryness *in vacuo* and yielded 2.42 g of semi-crystalline material. Catechol was removed by exhaustive extraction with dry benzene (4×40 ml.) in which hydroxyquinol is insoluble. Colour tests showed that the benzene extract contained no hydroxyquinol and on evaporation it yielded crystalline catechol. The residue (19 g) after benzene extraction gave intense colorations in the H_2SO_4 and 2,6-dichloroquinone chloroimide tests for hydroxyquinol, but attempts to obtain crystalline derivatives were unsuccessful. The extracted urine was now warmed to expel ether and then made 3N with respect to HCl. The mixture was boiled under reflux for 1 hr to hydrolyze glucuronides. After cooling, the urine was continuously extracted for 10 hr with peroxide free ether. The extract contained much catechol but no trace of hydroxyquinol. All extracts were also tested for pyrogallol without positive results.

It can, therefore, be concluded from these experiments that, in the rabbit, catechol is oxidized in small amounts to hydroxyquinol which appears in the ethereal sulphate, but not in the glucuronide, fraction of catechol urine.

DISCUSSION

It is clear from the above that the metabolism of catechol can be represented as follows



quinol can be detected readily in the presence of large amounts of catechol.

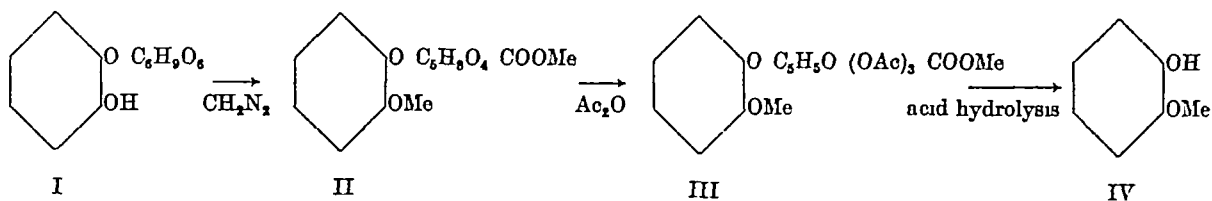
(ii) *The 2,6-dichloroquinone chloroimide test*. Traces of hydroxyquinol when added to 2-3 ml of a saturated aqueous solution of 2,6-dichloroquinone chloroimide gave a red colour which is intensified rapidly when the pH of the solution is raised to 8-9 by the addition of small amounts of $NaHCO_3$. This reagent also gives colours with phenol (blue) and catechol (blue purple), but the hydroxyquinol colour is always dominant in a mixture at pH 8-9 (Porteous & Williams, unpublished).

Eleven rabbits were fed a total of 6.36 g catechol (dose 200 mg/kg) and urine (2 l) collected over 24 hr. The urine was clarified by centrifuging and then made acid to litmus with a little dilute HCl. It was then continuously extracted for 5 hr with peroxide free ether. The extract contained catechol but no hydroxyquinol. From this we concluded that no hydroxyquinol was excreted in the free state. Ether was now expelled from the urine by gentle warming on the water bath, and the urine then was made \sim with respect to HCl and heated on a water bath for 0.5 hr to hydrolyze ethereal sulphates but not glucuronides. The product was now subjected to continuous extraction with

About 90% of catechol can be accounted for as catechol monoglucuronide, catechol monosulphuric acid, a small amount of free catechol and traces of conjugated hydroxyquinol, and these are excreted within 24 hr after dosing (see Fig 1).

The free catechol actually isolated as its crystalline *di p* toluenesulphonate was 1.8% of the dose and this must be considered as a minimum value owing to the losses inherent in the isolation of a small quantity of such a phenol. A figure of 5% of the dose would no doubt be nearer the true value for the free catechol excreted. The catechol glucuronide excreted has been proved to be a monoglucuronide. Methylation of the glucuronide (I) with diazomethane introduced two methyl groups thus indicating the presence of two acidic groups in the molecule, i.e. one free phenolic OH group and the carboxyl group of glucuronic acid. Methylation with diazomethane of the gummy catechol glucuronide did not yield a crystalline compound which could be characterized by melting point and optical rotation.

However, acetylation of the methylated glucuronide (II) yielded a crystalline product, viz *o*-methoxyphenyl 2,3,4-triacetyl β -D-glucuronide methyl ester (III). Hydrolysis of this ester produced guaiacol (IV) characterized as its *p*-toluenesulphonate. The isolation of guaiacol proves that the catechol glucuronide contains one free phenolic hydroxyl group and is therefore a monoglucuronide.



We have not provided direct proof that the ethereal sulphate excreted is a monosulphate but there is indirect evidence that this is so. Sulphuric esters of phenols are often extremely difficult to isolate from a complex fluid such as urine. Both the mono- and di-sulphuric esters of catechol have been prepared synthetically by Baumann (1878) who obtained the mono ester as a crystalline potassium salt and the di ester as an amorphous potassium salt. Neither of these esters has been isolated from biological sources (cf. Baumann & Herter, 1877-8). The mono ester gives a violet colour with ferric chloride and such a colour can be obtained with acetone extracts of catechol urine.

It can be argued on theoretical grounds that the formation of a disulphuric ester from the mono ester is unlikely, since catechol monosulphate is a phenol carrying a strongly acidic group in the *ortho* position. The situation is similar to that found in salicylic acid which does not form an ethereal sulphate in the rabbit (Williams, 1938). The reactivity of the free hydroxyl is suppressed for various reasons (*ortho* effect) by the adjacent strongly acidic group. Furthermore, Williams (1938) has shown that if, in an *o*-substituted phenol, the substituent group is a neutral one, e.g. the $-\text{OCH}_3$ group in guaiacol and the $-\text{CH}_3$ in *o*-cresol, then the extent of sulphate conjugation of that phenol is the same as found in phenol itself, i.e. c. 19% of the dose is excreted as sulphate. Assuming that catechol forms a monosulphate, the extent of its sulphate conjugation is 18% (see Table 1), in complete accord with the assumption that the second OH group is acting as a neutral group. There is also physico-chemical evidence which suggests that the reactivity of one hydroxyl group in catechol may be suppressed (e.g. Pauling (1945) on hydrogen bond formation in

catechol, see also Ball & Chen (1933) and Florence & Schapira (1943) on dissociation constants). It is interesting to note that there are many instances known of compounds carrying two or more similar groups capable of conjugation *in vivo* but in which conjugation occurs only on one of these groups, e.g. orcinol (Sera, 1913, 1914), phloroglucinol (Sera, 1914), phenolphthalein (Di Somma, 1940), stilb-

oestrol, hexoestrol and dienoestrol (Dodgson, Garton, Stubbs & Williams, 1948), etc.

Although the main fate of catechol in the rabbit involves conjugation only, it does undergo further oxidation to hydroxyquinol (1,2,4-trihydroxybenzene). Small amounts of conjugated hydroxyquinol were found in the urine, probably as an ethereal sulphate for it was only found in the ethereal fraction of the urine. From studies *in vitro* it has been shown that catechol is oxidized to hydroxyquinol by enzyme preparations containing catecholase (see review by Nelson & Dawson, 1944). Our results indicate that this oxidation also takes place in the intact animal. However, it is not clear from our experiments whether the hydroxyquinol arises by oxidation of free catechol or of a catechol conjugate. It is possible that it could arise by the oxidation of catechol monosulphate.

SUMMARY

1 The fate of catechol in the rabbit has been studied. At dose levels of 100 mg/kg, 70% of orally administered catechol is excreted in 24 hr conjugated with glucuronic acid and 18% conjugated with sulphuric acid. A small amount, c. 2%, is excreted in the free state. Some catechol is also oxidized to hydroxyquinol which appears in the urine as an ethereal sulphate.

2 The catechol glucuronide has been proved to be a monoglucuronide, by conversion into crystalline *o*-methoxyphenyltriacetylglucuronide methyl ester which on hydrolysis yields guaiacol. Evidence is also presented to show that the ethereal sulphate of catechol formed in the rabbit is a monosulphate.

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Metabolism of Derivatives of Toluene

1 THE METABOLISM OF ACETOTOLUIDIDES IN THE RABBIT

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In an investigation of the metabolism of the acetotoluidides, Jaffe & Hilbert (1888) found that all three isomers were oxidized in the dog and rabbit *p*-Acetotoluidide was shown to be converted almost quantitatively to *p* acetamidobenzoic acid in both animals, similarly, *m* acetamidobenzoic acid was isolated as a metabolite of *m* acetotoluidide and it was suggested that an aminocresol might also be formed *o* Acetotoluidide gave rise in the dog to a compound claimed to be a methylbenzoxazolone The constitution of this compound was not elucidated, but on treatment with ammonia in a sealed tube at 130-140° it gave a compound melting at 148-150° which appeared to be an aminocresol Seven amino cresols were known to Jaffe & Hilbert, but it is now apparent that the isomer they obtained was in fact 2 amino *m*-cresol which was not again described until 37 years later (Hodgson & Beard, 1925) The nature of the aminocresols formed as metabolites of acetotoluidides was of interest to us in view of the general rule concerning the position of entry of the hydroxyl group in biological hydroxylations which had been suggested by other investigations (e.g. Bray, Ryman & Thorpe, 1947, Bray, Lake, Neale, Thorpe & Wood, 1948) Consequently, we have carried out a further investigation of the fate of these compounds in the rabbit and included certain quantitative studies, the results of which are also reported here

EXPERIMENTAL

Materials and methods

Materials The acetotoluidides used in this study were prepared by the action of acetic anhydride upon the corresponding toluidines (Light and Co Ltd), the products being recrystallized from water The reference compounds, 2 amino 5 hydroxytoluene (6 amino *m* cresol) and 5 amino 2 hydroxytoluene (5 amino-*o* cresol), were prepared by the method of Noelting & Cohn (1884), which involves the reductive fission of azo compounds prepared from benzene-diazonium chloride and the appropriate cresol The azo compounds were dissolved in 2N NaOH and reduced by means of Na₂S₂O₄ The properties of the compounds obtained agreed with those described in the literature

Diet and feeding The rabbits used were does of 2-3 kg weight A constant diet of rabbit pellets, with water *ad lib*, was given throughout the study, as previously described (Bray *et al* 1947) The acetotoluidides were administered by stomach tube as suspensions in water The *p* isomer showed no toxic properties at a dose level of 0.5 g/kg, but, since at this dose level the *m* isomer was noticeably toxic and *o*-acetotoluidide slightly so, these two compounds were usually administered at a dose level of 0.25 g/kg

Estimation of diazotizable material This was carried out in acetotoluidide urines as collected, and after hydrolysis under the conditions described by Bratton & Marshall (1939) Compounds which might theoretically be included in the term 'diazotizable material' are aminocresols, with their glucuronides and ethereal sulphates, toluidines, and amino benzoic acids We found, however, that the two aminocresols

isolated as metabolites of *o* and *m* acetotoluidides gave no appreciable colour under the conditions of diazotization and coupling employed, and so made no significant contribution to the colour developed with either hydrolyzed or unhydrolyzed urine. We were not able to isolate glucuronides or ethereal sulphates of these compounds and their behaviour on diazotization and coupling is thus unknown. Since neither aminocresol is an *o*-aminophenol derivative, it would be expected that the colour given by the glucuronides or ethereal sulphates would be similar to that given by the parent compounds. However, these conjugates are found only in unhydrolyzed urine, and the amount of diazotizable material there is so small that it is in any case difficult to decide which compounds are responsible. In general, the colours obtained could be matched with relative ease against the colour developed from a standard solution of the corresponding aminobenzoic acid. The only diazotizable compounds, which are likely to make a contribution to the diazotizable material in hydrolyzed urine, are the toluidines and the aminobenzoic acids. The colours developed from these two types of compound cannot readily be matched, whereas the colours developed from hydrolyzed urine could in the majority of cases be readily compared with an aminobenzoic acid standard. This is indirect evidence for the absence of an appreciable amount of toluidines as is also the fact that there is reasonably good agreement between the results obtained from diazo estimations carried out on hydrolyzed urine and from estimations of ether soluble acid, which determine the total acetamidobenzoic acids present less the amount excreted as ester glucuronide. While these facts do not provide final proof that unchanged acetotoluidides (or toluidines) are not excreted as such, they strongly suggest that this is the case. Moreover, we were never able to isolate any of these compounds from acetotoluidide urines.

Aminobenzoic acid solutions were used as standards in all diazo estimations. A much longer time was required for maximum colour development with the *o* isomer than with the other compounds (Bray *et al* 1948).

Estimation of reducing substances The method used was that previously described (Bray, Neale & Thorpe, 1946a). The estimation was carried out only on unhydrolyzed urine, as aminocresols were found to reduce the Shaffer Hartmann reagent and, in the case of *p* acetotoluidide urines, we have found that the results obtained with urines passed after the administration of *p* aminobenzoic acid were unreliable (Bray *et al* 1948).

Estimation of ethereal sulphate The method of Folin (1905-6) was used as before.

Estimation of ether soluble acid The method used has already been described (Bray *et al* 1946a). Under these conditions acetamidobenzoic acids may be quantitatively extracted and estimated. Since it was found that no phenolic material (i.e. aminocresols or their acetyl derivatives) was extracted during this procedure, the results of these estimations may be taken as giving the total oxidation to acetamidobenzoic acid less that excreted as ester glucuronide. The errors involved, owing to the fact that unacetylated aminobenzoic acids are not quantitatively extracted at the pH used, are small since the amounts excreted are very small.

RESULTS

Quantitative studies

Excretion of normal metabolites On the diet used, it was found that the average daily excretion of ethereal sulphate ranged from 32 to 46 mg SO₃, of ether soluble acid (calculated as hippuric acid) from 709 to 913 mg, and of reducing material (calculated as glucuronic acid) from 224 to 307 mg.

Metabolism of p-acetotoluidide The results of the estimations performed on *p* acetotoluidide urines are summarized in Table 1. They strongly suggest that the oxidation to *p*-acetamidobenzoic acid is virtually complete, 96% of the dose being accounted for as

Table 1 *Metabolites of p-acetotoluidide in the rabbit*

Rabbit	Dose level (g/kg)	Percentage of dose excreted as				
		Ether soluble acid	Ester glucuronide	'Free' amino compounds	'Total' amino compounds	Ethereal sulphate
121	0.5	84	5.2	—	92	Nil
157	0.25	—	—	—	—	Nil
	0.5	—	4.8	0.3	—	—
	0.5	—	7.8	0.8	—	—
	0.5	78	11.6	0.9	100	—
162	0.5	—	9.8	1.0	—	—
	0.5	—	7.0	0.9	—	—
163	0.25	—	8.4	—	—	Nil
	0.5	—	12.0	1.0	—	—
	0.5	—	7.7	0.8	—	—
	0.5	80	12.8	1.3	100	—
165	0.5	77	6.3	0.4	83	—
	0.5	80	—	1.1	100	—
166	0.5	77	9.4	0.3	94	—
170	0.5	78	17.9	0.3	95	—
	0.5	—	20.8	0.9	100	—
Average	—	79	10.0	0.8	96	Nil

this compound by diazo estimation, and 88% as the sum of the ether soluble acid (78%) and ester glucuronide (10%) *p*-Acetotoluidide causes no increase in ethereal sulphate excretion, as we anticipated, since Hammerbacher (1884) found the same for *p* toluidine in the dog, and we showed that *p*-aminobenzoic acid had no effect upon ethereal-sulphate excretion in the rabbit (Bray *et al* 1948) The amount of deacetylation which occurs is very small

estimation Attempts to isolate this compound have been unsuccessful, possibly because of such instability A similar result was observed in the case of sulphapyridine, where there is no possibility of the formation of an ester glucuronide, and yet unhydrolyzed sulphapyridine urine had a reducing value greater than normal It is possible that this may have been due to either (a) the instability of the glucuronide of hydroxysulphapyridine, a metabolite of the drug (Bray, Neale & Thorpe, 1946b),

Table 2 *Metabolites of m-acetotoluidide in the rabbit*

Rabbit	Dose level (g/kg)	Percentage of dose excreted as			
		Ether soluble acid	'Free' amino compounds	'Total' amino compounds	Ethereal sulphate
121	0.25	36	0.3	23	—
	0.25	34	Tr	27	—
153	0.25	30	—	25	—
	0.25	34	—	—	—
154	0.25	31	—	22	—
	0.25	19	—	26	—
157	0.25	49	Tr	28	9
	0.25	48	Tr	26	—
	0.25	42	—	—	—
163	0.25	29	Tr	25	10
	0.25	27	Tr	30	—
	0.25	27	—	—	—
173	0.25	24	Tr	18	10
	0.25	37	Tr	27	—
	0.25	31	—	—	—
	0.25	27	—	—	—
Average	—	34	Tr	25	10

Metabolism of m-acetotoluidide Table 2 summarizes the results of estimations carried out on the urine of rabbits which had received *m* acetotoluidide Since it is concluded that the results obtained from the estimation of total diazotizable material are due entirely to *m* aminobenzoic acid, 25% of the dose appears to be oxidized to this compound, a value agreeing reasonably well with that obtained from the estimation of ether soluble acid (34%) The value obtained from the diazo estimation is probably the more accurate since it does not involve 'baseline' values The results of the estimations of reducing material are difficult to interpret and are not included in Table 2 In ten experiments the percentage of *m* acetotoluidide excreted, apparently conjugated as ester glucuronide, ranged from 10 to 25 (average 16%) It is unlikely that this is a true value, since the diazo values should indicate the total extent of oxidation to *m* acetamidobenzoic acid, i.e. should approximate to ether soluble acid plus ester glucuronide Three reasons may be suggested for the high reducing values obtained, but we have been unable to obtain direct proof of any of them (i) It is possible that the glucuronide of 5 amino *m* cresol may be unstable under the conditions used for

which might break down to glucuronic acid and hydroxysulphapyridine, which is itself reducing, or (b) reducing properties of the glucuronide itself, though these may have been due to (a) It may be significant that we were not able to isolate this glucuronide, in order to determine whether these suggestions have any foundation in fact (ii) The second reason, which may be suggested to account for the high reducing values, is that *m* acetotoluidide might have some effect upon carbohydrate metabolism A similar effect was observed in connexion with *o* acetotoluidide (see p. 214), but we have been unable so far to study the problem further (iii) 5 Amino *o* cresol reduces the Shaffer Hartmann reagent, but, since we have been unable to detect the free compound in *m* acetotoluidide urines, it seems likely that it is only excreted in conjugated form

Metabolism of o-acetotoluidide This study gave results qualitatively similar to those on the *m*-isomer It may be concluded from the diazo estimations (Table 3) that 6% of the dose is oxidized to anthranilic acid This agrees reasonably well with the results obtained from the determinations of ether soluble acid, though the latter cannot be regarded as precise since 8% of a dose of 600 mg

Table 3 *Metabolites of o acetotoluidide in the rabbit*

Rabbit	Dose level (g/kg)	Percentage of dose excreted as			
		Ether soluble acid	'Free' amino compounds	'Total' amino compounds	Ethereal sulphate
121	0.25	3.6	7.2	6.4	28
	0.25	3.4	5.2	6.2	—
153	0.25	6.9	5.7	5.9	—
	0.25	6.8	5.0	6.5	—
154	0.25	18.7	4.7	6.3	—
	0.25	16.8	4.8	5.8	—
157	0.25	—	—	—	30
163	0.25	—	—	—	37
173	0.25	8.5	8.2	7.5	—
	0.25	7.1	5.5	6.9	—
	0.25	16.0	4.5	5.1	—
Average	—	7.5	5.6	6.3	32

corresponds to an amount of acid (58 mg hippuric acid) much less than the variations which occur in the normal 'base line' values. Indeed, the excretion of ether-soluble acid in the 24 hr period after the administration of *m*-acetotoluidide was in some cases actually within the normal range. Estimations of reducing value carried out on unhydrolyzed *o*-acetotoluidide urine gave results corresponding to conjugation of 25–68% (average 42%) of the dose as ester glucuronide. It seems unlikely, from the agreement between the results of the diazo estimations and determinations of ether soluble acid, that the reducing value actually represents ester-glucuronide excretion, and the explanations suggested in the case of the *m* isomer may apply here also. Free 6-amino *m*-cresol was not detected in *o*-acetotoluidide urines. We were also unable to isolate a glucuronide in order to test the hypothesis based on its instability. The fact that in one rabbit the increased excretion of reducing material (approximately twice the normal value) persisted for 4 days after dosing is in accordance with the suggestion that the high reducing values observed are due to some more general influence upon metabolism, e.g. of carbohydrate. In other rabbits the excretion of reducing material was restored to normal during the second 24 hr. We were unable to determine the nature of the reducing material excreted. In none of the rabbits was it accompanied by ketonuria, nor was it fermentable by yeast. We were unable to isolate a crystalline phenylosazone. There was little difference between the diazo results obtained with unhydrolyzed and hydrolyzed urine. The percentage of the dose excreted unacetylated was greater with *o*-acetotoluidide than with the other isomers. When anthranilic acid itself is administered, no acetylation appears to occur (Bray *et al.* 1948). A considerable percentage of *o*-acetotoluidide is hydroxylated and excreted conjugated with sulphate. This is in agreement with

Hammerbacher's (1884) observation that *o*-toluidine caused an increase in excretion of ethereal sulphate in the dog.

Qualitative studies

o-Acetotoluidide. The urine of rabbits which had received this compound (1 g each) was collected for 24 hr after dosage, acidified with conc. HCl (0.1 vol) and continuously extracted with ether for 24 hr. In this way a small amount of syrupy material (1 g from urine of five rabbits) was obtained. This gave a slightly positive diazo reaction, which was much more intense after acid hydrolysis. From the syrup a small amount (10 mg) of crystalline material (m.p. 184°) was isolated, and shown to be identical with authentic acetylanthranilic acid (m.p. 185°).

The urine was then acidified further with 0.1 vol conc. HCl and hydrolyzed by boiling for 1 hr. The pH was adjusted to 8 and the urine continuously extracted with ether for 24 hr. A dark syrup was obtained which gave an intense red-purple colour with ferric chloride. Purification by dissolving in water, treatment with charcoal and evaporation of the filtrate gave colourless needles, darkening in air, m.p. 178° alone or mixed with 6-amino *m*-cresol (m.p. 178°). This compound gives, on diazotization and coupling with dimethyl- α -naphthylamine or *N*-(1-naphthyl) ethylenediamine dihydrochloride, a transient blue colour, but on long standing a violet colour develops. These colours are feeble compared with those obtained from aminobenzoic acids and in solutions of 10–20 mg/100 ml are almost negligible. The yield of 6-amino *m*-cresol cannot be definitely stated. Most procedures for isolation yielded about 100 mg from a 1 g dose, but owing to the great sensitivity of the compound to oxidation by air this does not represent the entire yield. In view of its instability it is reasonable to suggest that it is actually excreted as its *N*-acetyl derivative, though

we could not prove this directly, owing to its feeble diazotization and poor coupling properties. No free aminocresol was detected and no other metabolite was isolated.

o Acetotoluidide urine gave a strongly positive naphthoresorcinol reaction and attempts were made to isolate a glucuronide (cf Bray *et al* 1947). These yielded only syrups which could not be crystallized, but from which, after hydrolysis, 6 amino *m* cresol was isolated.

m Acetotoluidide. The method used for the isolation of metabolites of this compound was essentially the same as with the *o* isomer. Extraction of the acidified urine yielded crystalline *m* acetamidobenzoic acid which was purified by treatment with charcoal and recrystallization from water. It melted at 248° alone or mixed with an authentic specimen (m.p. 248°), yield, 20% of the dose. Extraction of the hydrolyzed urine gave a crystalline product, which after recrystallization from benzene had m.p. 174° alone or mixed with synthetic 5 amino *o* cresol. Its identity was further confirmed by comparison of acetyl derivatives obtained by the action of acetic anhydride at ordinary temperature. These compounds melted at 178° alone or mixed together. Yield, 30% of the dose. As with the aminocresol from *o* acetotoluidide, it is probable that the acetyl derivative is the compound actually excreted.

p Acetotoluidide. Extraction of acidified unhydrolyzed urine enabled 70% of the dose to be recovered as crystalline *p* acetamidobenzoic acid. No other metabolite was isolated.

DISCUSSION

It can be seen that the results obtained in this investigation confirm in general those of Jaffe & Hilbert (1888). In addition we were able to show the formation from *o* acetotoluidide of anthranilic acid, of which only small part is excreted acetylated. 5 Amino *o* cresol was isolated from hydrolyzed *m*-acetotoluidide urine. In the rabbit *o* acetotoluidide gives rise chiefly to 6 amino *m* cresol, whereas it now appears that Jaffe & Hilbert found 2 amino *m*-cresol to be the chief metabolite in the dog. It is of interest that we obtained oxidation products of both these types from *o* aminobenzoic acid in the rabbit

(Bray *et al* 1948), but we were unable to detect the vicinally substituted compound here. The configuration of the three aminocresols obtained by us, or by Jaffe & Hilbert, are in accordance with the general rule that the entering hydroxyl group takes up a position ortho or para to the amino (or acetamido) group already present (see Bray *et al* 1948).

The formation of benzoxazolone or its derivatives *in vivo* is of interest. To our knowledge only three such cases have been described, viz. from acetanilide and *o* acetotoluidide (Jaffe & Hilbert, 1888), and from formanilide (Kleine, 1896-7). In all these studies the experimental animal was the dog. It would be of interest to determine whether any other animal is capable of ring formation of this type, since *o* aminophenol compounds are common metabolites of amino compounds. There is a possibility that the benzoxazolone derivatives formed may be artifacts, since it is known that *o* aminophenol (or its hydrochloride) and urea give rise to benzoxazolone when heated together at a high temperature (Sandmeyer 1886, Williams, 1947). In all three instances where benzoxazolone was isolated the urine was first evaporated to dryness. Whether this treatment is drastic enough to deacetylate the *o* aminophenol derivative and then condense it with urea appears doubtful, but this question is under investigation.

The quantitative results show an interesting gradation in metabolic behaviour, as can be seen in Table 4.

It is probable that the *N* acetyl group in *p* acetotoluidide remains intact throughout metabolism, since the degree of acetylation of the *p* aminobenzoic acid excreted (at least 88%) is very much greater than when this acid is itself ingested (4-21%, Bray *et al* 1948). It is not possible to say, with any certainty, whether this applies also to the other acetotoluidides, since other metabolites are also formed.

SUMMARY

1. The metabolism of *o*, *m* and *p* acetotoluidides in the rabbit has been studied.

2. *o* Acetotoluidide is partly oxidized to anthranilic acid (6%) and acetyl anthranilic acid (1%), a considerable part of the remainder appears to be converted to 6 amino *m* cresol, which has been isolated. This latter compound is probably excreted as its

Table 4. Summary of metabolites of acetotoluidides in the rabbit

Isomer	Average percentage of dose excreted as				
	Ether soluble acid	Ester glucuronide	'Free' diazotizable material	'Total'* diazotizable material	Ethereal sulphate
ortho	8	—	5.6	6	32
meta	34	—	Tr	25	10
para	79	10	0.8	96	Nil

* Assumed equivalent to total oxidation of CH₃ group

N-acetyl derivative conjugated with glucuronic acid and sulphate. The extent of sulphate conjugation corresponds to 32 % of the dose.

3 The metabolism of *m*-acetotoluidide is similar to that of the ortho isomer, 25 % is oxidized to the corresponding *N*-acetamidobenzoic acid, and the only other metabolite detected was 5 amino *o* cresol which has been isolated. This is probably excreted as its *N* acetyl derivative conjugated with glucuronic acid and sulphate, 10 % of the dose is excreted as an ethereal sulphate.

4 *p* Acetotoluidide is virtually completely oxidized to *p* acetamidobenzoic acid, 10 % being excreted as ester glucuronide. There is no conjugation with sulphuric acid.

5 *o* and *m* acetotoluidides cause an abnormally high excretion of reducing material in urine. The reason for this has not been determined.

The sulphate estimations were carried out by Mr P B Wood, to whom the authors' thanks are due. We are also indebted to the Royal Society for a Government Grant which defrayed part of the cost of the investigation.

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Studies in the Biochemistry of Micro-organisms

78 THE MOLECULAR CONSTITUTION OF MYCOPHENOLIC ACID, A METABOLIC PRODUCT OF *PENICILLIUM BREVI-COMPACTUM* DIERCKX

PART 2 POSSIBLE STRUCTURAL FORMULAE FOR MYCOPHENOLIC ACID

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Mycophenolic acid, $C_{17}H_{20}O_6$, was first named and isolated from cultures of *Penicillium stoloniferum* Thom by Alsberg & Black (1913), although Gosio (1896) had reported an unnamed crystalline metabolic product of '*P. glaucum*', to which he assigned the formula $C_9H_{10}O_3$, which is probably identical with mycophenolic acid. Clutterbuck, Oxford, Raistrick & Smith (1932) isolated mycophenolic acid, together with other phenolic compounds, from the culture filtrates of twelve species or strains in the *P. brevis compactum* series, to which *P. stoloniferum* Thom belongs, and Clutterbuck & Raistrick (1933) reported an extensive chemical investigation of this substance.

Gosio (1896) demonstrated that his substance, $C_9H_{10}O_3$, suppresses the growth of the anthrax bacillus, so that it appears probable that mycophenolic acid, although of relatively low potency, was the first antibiotic of fungal origin to be obtained

in crystalline form. More recently, Florey, Gilliver, Jennings & Sanders (1946) investigated its antibacterial action against a number of animal and plant pathogens and also against a number of saprophytic and pathogenic fungi. They showed that it is more effective against Gram positive than against Gram negative bacteria and that its activity against staphylococci varies greatly with the number of organisms used for the inoculum. This 'inoculum size' phenomenon was thoroughly investigated by Abraham (1945).

Clutterbuck & Raistrick (1933) established, *inter alia*, the following facts bearing on the constitution of mycophenolic acid.

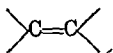
(1) It is optically inactive.

(2) The six oxygen atoms are present in the molecule in the following forms: (a) One as a OCH_3 group. On demethylation, normycophenolic acid, $C_{16}H_{18}O_6$, is formed. (b) One as a phenolic OH group, re-

sponsible for the blue violet FeCl_3 colour (c) Two as a COOH group (d) Two as a lactone ring,

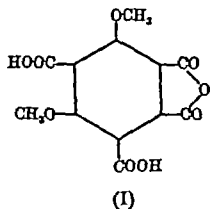


(3) It contains the aliphatic grouping

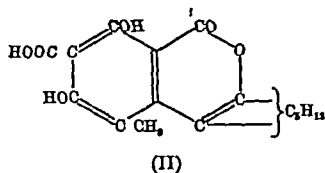


(4) On fusion with KOH , 1 5 dihydroxy 3 4 dimethylbenzene is formed

(5) Its monomethyl ether, on protracted oxidation with alkaline KMnO_4 , gives the anhydride of 1 5-dimethoxy 2 3 4 6 tetracarboxybenzene (I) in good yield.



On the basis of these facts, and on others for which the original paper should be consulted, the structure (II) for normycophenolic acid was advanced



No evidence was available as to which of the two phenolic groups in (II) is methylated in mycophenolic acid itself

We now present experimental evidence which necessitates modification and amplification of structure (II) and may be summarized as follows

I Ozonization of mycophenolic acid, its monomethyl ether, and its monomethyl ether methyl ester

Chloroform solutions of these three substances were ozonized, and, after decomposing the resulting ozonides with water, the following breakdown products were isolated

(a) From mycophenolic acid ($\text{C}_{17}\text{H}_{20}\text{O}_6$, with 1 OCH_3 group) levulic acid, $\text{CH}_3\text{COCH}_2\text{CH}_2\text{COOH}$, and an aldehyde $\text{C}_{12}\text{H}_{12}\text{O}_5$, containing 1 OCH_3 group, giving the same FeCl_3 reaction as the parent substance, and not extractable from chloroform with aqueous NaHCO_3

(b) From mycophenolic acid monomethyl ether ($\text{C}_{18}\text{H}_{22}\text{O}_6$, with 2 OCH_3 groups) levulic acid, an aldehyde, $\text{C}_{13}\text{H}_{14}\text{O}_5$, containing 2 OCH_3 groups, which will be dealt with more fully later, and a small

amount of the corresponding carboxylic acid, $\text{C}_{13}\text{H}_{14}\text{O}_6$

(c) From mycophenolic acid monomethyl ether methyl ester ($\text{C}_{19}\text{H}_{24}\text{O}_6$, with 3 OCH_3 groups) methyl levulate, $\text{CH}_3\text{COCH}_2\text{CH}_2\text{COOCH}_3$, and the same aldehyde $\text{C}_{13}\text{H}_{14}\text{O}_5$, containing 2 OCH_3 groups, as was obtained by ozonization of mycophenolic acid monomethyl ether

These findings clearly prove that mycophenolic acid contains (1) a hydroxyl group, directly attached to the nucleus, which can be methylated by diazomethane and is therefore phenolic in nature, and (2) a free carboxyl group in a side chain which gives rise to levulic acid on ozonolysis of mycophenolic acid or its monomethyl ether and to methyl levulate on similar treatment of its monomethyl ether methyl ester

The free COOH group attached directly to the nucleus in structure (II) must, therefore, now be transferred to the end of a side chain of six or more carbon atoms including the C_6H_{12} residue, the nature of which was left undetermined by Clutterbuck & Raistrick (1933)

II Molecular constitution of the aldehyde, $\text{C}_{13}\text{H}_{14}\text{O}_5$

The substance, $\text{C}_{13}\text{H}_{14}\text{O}_5$, obtained by the ozonolysis of both the monomethyl ether and the monomethyl ether methyl ester of mycophenolic acid, is a neutral substance and is a true aldehyde and not a ketone. It gives typical aldehyde reactions, is oxidized by alkaline iodine to a carboxylic acid, $\text{C}_{13}\text{H}_{14}\text{O}_6$, and on heating with acetic anhydride and anhydrous sodium acetate it gives a mono enol acetate. The important conclusion follows that in the aldehyde, $\text{C}_{13}\text{H}_{14}\text{O}_5$, the CHO group is not attached directly to the nucleus but through an intervening $-\text{CH}_2-$ link, the acetylated product having the form RCHCHOCOCH_3 . This conclusion is confirmed by the fact that ozonolysis of the enol acetate yields an aldehyde, $\text{C}_{12}\text{H}_{12}\text{O}_5$, fission having occurred at the double linking

Of the thirteen carbon atoms present in the aldehyde, $\text{C}_{13}\text{H}_{14}\text{O}_5$, six are present in a benzene ring, since permanganate oxidation of mycophenolic acid monomethyl ether yields the anhydride of 1 5-dimethoxy 2 3 4 6 tetracarboxybenzene, structure (I), two as OCH_3 groups and two as the group $-\text{CH}_2\text{CHO}$, leaving three carbon atoms to be accounted for. It follows from the structure of the above anhydride (I) that each of these three carbon atoms must be attached singly to the benzene nucleus

The nature of the groups in which each of these three carbon atoms is present may now be considered. The aldehyde $\text{C}_{13}\text{H}_{14}\text{O}_5$ (A) behaves as a neutral substance on titration with NaOH in the cold, but as a monobasic acid on heating. It is thus

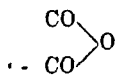
probably a monolactone. Alkaline iodine converts it into a compound $C_{13}H_{14}O_6$ (B) which still retains two methoxy groups but has lost its aldehydic properties. It titrates in the cold as a monobasic acid and on heating as a dibasic acid. It is therefore a monobasic acid monolactone. The disodium salt of (B), on oxidation with cold neutral aqueous $KMnO_4$, yields a substance which, after sublimation in a high vacuum, has the empirical formula $C_{13}H_{12}O_7$ (C). This substance still retains both OCH_3 groups, and titrates sharply in the cold as a tribasic acid. These facts can be most simply explained by postulating that (A) contains the groups $HCO-CH_2-$ and



that in (B) the $HCO-CH_2-$ group becomes $HOOC-CH_2-$ and the lactone grouping remains unchanged, that (C) still retains the group $HOOC-CH_2-$ while the lactone ring is opened in the sodium salt to



and oxidized and the ring reclosed on sublimation to give the anhydride ring

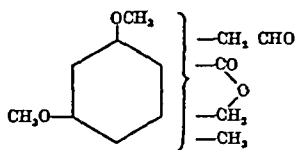


Thus we may conclude that two of the three carbon atoms in (A), whose nature is in doubt, are vicinal and in the form

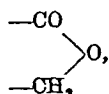


The third carbon atom is present as a CH_3 group, since the aldehyde $C_{13}H_{14}O_5$ (A) was shown by analysis to contain one carbon-methyl, $CH_3(C)$, group. Mycophenolic acid itself was shown to contain two carbon-methyl groups, the second one being the branched CH_3 group in the side chain in structures (IX) and (X).

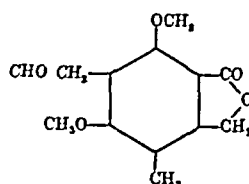
Since the two methoxyl groups are attached directly to the benzene nucleus in the meta position to each other (Clutterbuck & Raistrick, 1933) the constitution of the $C_{13}H_{14}O_5$ aldehyde may therefore be written in the form



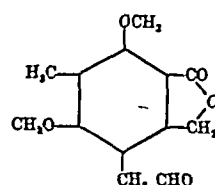
and since each of the four different substituent groups, two of them, viz



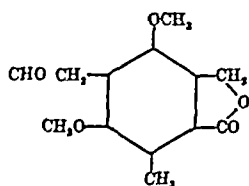
vicinal, must be attached directly to the nucleus, there are only four possible isomers, (III), (IV), (V) and (VI)



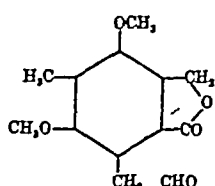
(III)



(IV)



(V)



(VI)

Evidence as to which of these four structural formulae most probably represents the structure of the aldehyde $C_{13}H_{14}O_5$, and hence part of the structure of the monomethyl ether of mycophenolic acid from which it is derived by ozonization, is available from a number of sources, all of which lead to the same conclusion

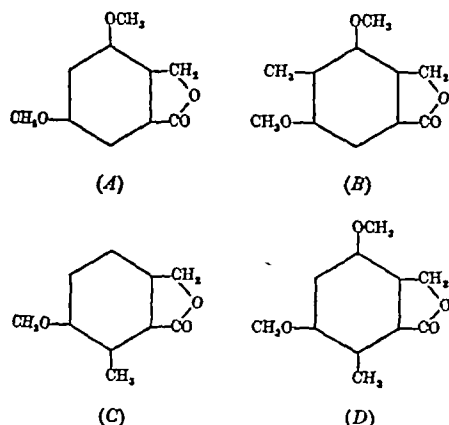
(a) On the addition of a suspension of 2,6-dichloroquinone chloroimide to a solution of normycophenolic acid in sodium borate buffer of pH 9.2, at which pH the phthalide ring will be opened and a $COOH$ group liberated, a blue colour with a tinge of green is rapidly formed. The rapid production of a blue colour by a substituted benzene compound is indicative, according to Gibbs (1927), of a free position para to a phenolic group, though according to Davidson, Keane & Nolan (1943) the reaction is also positive if the position para to OH is occupied by $COOH$. Examination of structures (III), (IV), (V) and (VI), assuming the OCH_3 groups to be replaced by OH groups as in normycophenolic acid, shows that only (III) and (IV) satisfy the requirement of $COOH$ para to OH .

(b) Clutterbuck & Raistrick (1933) showed that, on fusion with KOH , mycophenolic acid yields 1,5-dihydroxy-3,4-dimethylbenzene. Hence, if one ignores the possibility of migration of substituents during potash fusion, two of the three substituents, CH_3 , CH_2OH and CH_2CHO , likely to give rise to the two CH_3 groups in the fusion product, must be vicinal. This requirement also is satisfied only by (III) and (IV).

(c) One of the groups shown as methoxyl in (V) and (VI) is represented by a free (phenolic) OH in mycophenolic acid itself. As both methoxyl groups of (V) and (VI) are meta to the potential carboxyl group, the OH group of mycophenolic acid must also

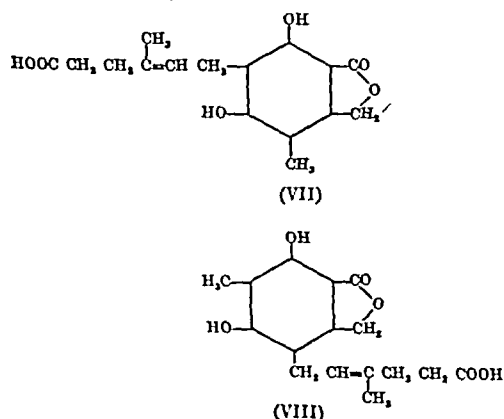
be meta to the potential carboxyl if either (V) or (VI) is correct. This orientation would be unlikely to give rise to the intense *violet* coloration which mycophenolic acid gives with ferric chloride, whereas the possible *o* OH group derivable from (III) and (IV) would be expected to give such a colour.

(d) Of the four known phthalides



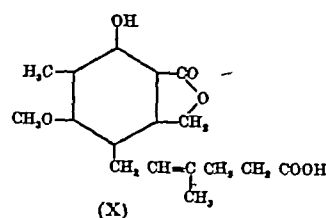
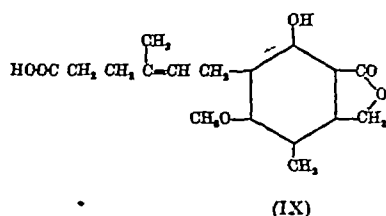
(A) (Fritsch, 1897) and (B) (Charlesworth & Robinson, 1934) are readily oxidizable to the corresponding phthalic acid by means of alkaline permanganate, whereas (C) (Charlesworth, Rennie, Sinder & Yan, 1945) and (D) (Mittar, Sen & Paul, 1927, Charlesworth *et al.* 1945) are not so oxidized. It appears that the presence of a substituent methyl group ortho to the potential carboxyl of the phthalide grouping prevents oxidation. If this is the case it would rule out formulae (V) and (VI) as possibilities for the C_{13} aldehyde, since (V) has a methyl group and (VI) a substituted methyl group ortho to the potential carboxyl, yet the product, after oxidation of $-CHO$ to $-COOH$ was oxidized by permanganate to the phthalic acid.

The two most probable formulae for normycophenolic acid therefore become (VII), from (III), and (VIII), from (IV).



We have no experimental evidence capable of deciding between (VII) and (VIII), but we are inclined to favour (VII), since this structure contains a preformed CH_3 group oriented towards the two OH groups as in 1,5-dihydroxy-3,4-dimethylbenzene, the potash fusion product of mycophenolic acid.

Finally, there arises the question as to which of the two OH groups in (VII) or (VIII) is methylated in mycophenolic acid itself. We have stated previously that normycophenolic acid rapidly gives a blue colour with 2,6-dichloroquinone chloroimide. Under the same conditions mycophenolic acid gives only a very faint dirty yellow colour even on standing. We are aware of the dangers of interpreting a negative 2,6-dichloroquinone chloroimide reaction (see Calam, Clutterbuck, Oxford & Raistrick, 1947), but, with this reservation, the choice of the hydroxyl group to be represented as methylated in mycophenolic acid has fallen on that para to a potential carboxyl group. This choice is strengthened by the fact that an aqueous solution of mycophenolic acid gives with aqueous $FeCl_3$ a strong violet colour somewhat similar to, but admittedly bluer than, that given by salicylic acid. Hence we believe that the more probable structure for mycophenolic acid is (IX) and the less probable (X).



It remains now to show that the experimental evidence obtained by Clutterbuck & Raistrick (1933) can be explained by the favoured constitution for mycophenolic acid. In most cases this is self-evident, but two points call for further discussion.

Titration values. On titration to phenolphthalein in water with aqueous NaOH in the cold, mycophenolic acid and its derivatives gave the following titration equivalents:

(a) Normycophenolic acid, 155.6 Theor. as a dibasic acid, 153.

(b) Mycophenolic acid, 169 Theor. as a dibasic acid, 160.

(c) Mycophenolic acid monomethyl ether, 332 Theor as a monobasic acid, 334

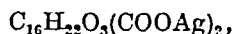
(d) Monoacetylmycophenolic acid, 361 Theor as a monobasic acid, 362

On heating the cold titrated solutions with excess of NaOH and back titrating, mycophenolic acid and its monomethyl ether became fully dibasic, while acetylmycophenolic acid became tribasic

The interpretation of these findings appears to be as follows

(1) Monoacetylmycophenolic acid is completely hydrolyzed with the formation of one molecule each of acetic and mycophenolic acids

(2) Mycophenolic acid monomethyl ether Since there is no free phenolic OH group in this compound either before or after hydrolysis, the ether being recovered unchanged from the acidified hydrolysis solution, it is clear that the free carboxyl group at the end of the long substituent chain titrates in the cold and that, on heating, the phthalide ring is opened and the COOH group thus liberated then titrates The formation of a disilver salt,

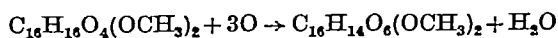


involving the addition of the elements of water confirms this view

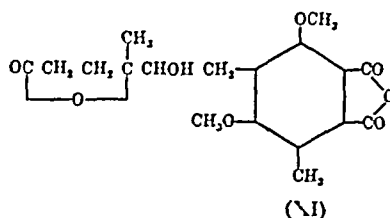
(3) Mycophenolic acid Two explanations seem possible (a) One equivalent of acid is due to the free terminal COOH group The second (partial) equivalent in the cold is due to the partial, but almost complete, opening of the phthalide ring in the cold This, however, is contrary to the usual behaviour of the phthalide grouping (b) An alternative explanation is that the second (partial) equivalent in the cold is due to the acidity of the phenolic OH group It must then be assumed that, on hot alkaline hydrolysis, the phthalide ring is opened, and that the resulting formation of a nuclear carboxyl group has suppressed the ionization of the phenolic OH group, so that the latter has no longer a titratable acidity This view is confirmed by the fact that according to Alsberg & Black (1913) mycophenolic acid forms two barium salts having the empirical formulae $(\text{C}_{17}\text{H}_{18}\text{O}_6)_2\text{Ba}$ and $\text{C}_{17}\text{H}_{18}\text{O}_6\text{Ba}$ The latter dibasic salt clearly arises without the addition of the elements of water, and hence, inferentially, without the opening of the phthalide ring

The constitution of the alkaline permanganate oxidation product of mycophenolic acid monomethyl ether (Clutterbuck & Raistrick, 1933, p 659)

This substance, which was isolated in good yield, has the empirical formula $\text{C}_{16}\text{H}_{14}\text{O}_6(\text{OCH}_3)_2$ It has, therefore, the same number of carbon atoms as the ether from which it is formed according to the equation



Since, unlike the parent ether, it does not immediately decolorize KMnO_4 in the cold, it probably arises by oxidation at the double bond to the glycol form (requiring one O), followed by lactonization on subsequent isolation in acid solution, coupled with oxidation of the phthalide to the phthalic anhydride (requiring two O), the product having the constitution (XI) from structure (IX) or the corresponding structure derived from (X)



It was found that the oxidation product, when dissolved in ethanol, titrated in the cold as a monobasic acid, but after alkaline hydrolysis as a tribasic acid A product of structure (XI), when dissolved in ethanol, should yield the mono acid mono ester derived from the acid anhydride ring This would then titrate in the cold as a monobasic acid, whereas two new acidic groups would be revealed on alkaline hydrolysis, one by hydrolysis of the ester grouping and the other by the opening of the lactone ring This would also explain the fact that the substance was recovered unchanged after acidifying the hydrolysis solution

EXPERIMENTAL

A. Action of ozone on mycophenolic acid and its derivatives

In this series of experiments ozonized O_2 was passed through a chloroform solution of the substance until a sample of the solution no longer decolorized a dilute solution of Br_2 in chloroform The chloroform solution of the ozonide was well shaken with its own volume of water, warmed to $c\ 50^\circ$ and again thoroughly shaken to decompose the ozonide After cooling, the water fraction was separated from the chloroform fraction and the two were worked up separately

I Action of ozone on mycophenolic acid Mycophenolic acid (0.5 g) in chloroform (25 ml) was ozonized for 2.5 hr

Water fraction The solution was treated with NaHCO_3 (0.5 g) and continuously extracted with ether On removal of the solvent only a trace of whitish residue remained The extraction was continued after acidification with $2\text{N-H}_2\text{SO}_4$ The oil (0.18 g) thus obtained was treated with 50 ml. of Brady's reagent (0.3% 2,4-dinitrophenylhydrazine in 2N-HCl) An orange yellow crystalline precipitate soon formed, m.p. $203\text{--}205^\circ$, raised to 205° after recrystallization from methanol, and not depressed on admixture with authentic levulinic acid 2,4-dinitrophenylhydrazone, m.p. $205\text{--}206^\circ$ The substance is acid to litmus, and is readily soluble in aqueous NaHCO_3 to a golden yellow solution from which it separates on acidification as an orange flocculent precipitate

Chloroform fraction The chloroform solution was washed with aqueous NaHCO_3 . The acids were liberated by acidification with $2N \text{H}_2\text{SO}_4$ and extracted with ether, yielding 0.06 g of colourless crystalline solid which was not further examined. The neutral fraction, after removal of chloroform, remained as a mixture (0.21 g) of crystals and oil. It was washed with a little ether to remove the oil, and the remaining crystals, after recrystallization first from ether and then from water, gave 0.07 g of short colourless rods, m.p. 152–153° (Found C, 61.23, 61.14, H, 5.12, 5.40, OCH_3 , 12.7, 13.3 $\text{C}_{11}\text{H}_{14}\text{O}_4(\text{OCH}_3)_2$ requires C, 61.01, H, 5.12, OCH_3 , 13.1%) The substance gave a strong aldehyde reaction (Schiff), and gave in water and in ethanol with FeCl_3 the same colour reactions as mycophenolic acid. It dissolved readily in cold $2N \text{NaOH}$ giving an immediate yellow colour which became somewhat darker on standing. The substance is therefore an aldehyde, $\text{C}_{12}\text{H}_{12}\text{O}_5$, with one methoxyl group, and evidently the grouping, a phenolic OH group, responsible for the FeCl_3 reaction with mycophenolic acid, persists in its ozonolysis product.

II. Action of ozone on mycophenolic acid monomethyl ether Mycophenolic acid monomethyl ether (1.0 g, m.p. 112°, see Clutterbuck & Raistrick, 1933, p. 658) in chloroform (50 ml) was ozonized for 2.25 hr. A total of 5.30 g of the ether was ozonized.

Water fraction The combined water fractions were treated with an excess of Brady's reagent giving an orange crystalline precipitate, 1.16 g, m.p. 201–203° of levulinic acid 2,4-dinitrophenylhydrazone. A sample recrystallized from methanol in orange needles, m.p. 204–205°, was analyzed. (Found C, 44.38, H, 4.63, N, 19.05 $\text{C}_{11}\text{H}_{12}\text{O}_6\text{N}_4$, i.e. the dinitrophenylhydrazone of $\text{C}_6\text{H}_5\text{O}_3$, requires C, 44.60, H, 4.08, N, 18.9%.)

Chloroform fraction The combined chloroform extracts were washed twice with 50 ml. aqueous NaHCO_3 , and this extract on acidification yielded 0.39 g of almost colourless crystals with some oil. On recrystallization from chloroform—light petroleum colourless needles were obtained, m.p. 153°, identical with the alkaline iodine oxidation product of the aldehyde $\text{C}_{12}\text{H}_{14}\text{O}_5$ (Found C, 59.05, 59.20, H, 5.17, 5.10 $\text{C}_{12}\text{H}_{14}\text{O}_5$ requires C, 58.64, H, 5.30%.)

The bicarbonate-extracted chloroform solution, on removal of the solvent, yielded an oil which soon crystallized. On recrystallization from water a colourless crystalline solid, 2.05 g, of constant m.p. 112° was obtained. Concentration of the aqueous mother liquors gave a further 0.4 g, m.p. 107°. The substance gives a strong Schiff reaction and is the same aldehyde, $\text{C}_{12}\text{H}_{14}\text{O}_5$, m.p. 112° as was obtained by the ozonization of mycophenolic acid monomethyl ether methyl ester (see section AIII).

Mono-2,4-dinitrophenylhydrazone of the aldehyde $\text{C}_{12}\text{H}_{14}\text{O}_5$ An ethanol solution of the $\text{C}_{12}\text{H}_{14}\text{O}_5$ aldehyde on treatment with Brady's reagent gave an immediate precipitate which on recrystallization from methanol yielded deep yellow needles, m.p. 214° (Found C, 53.42, H, 4.34, N, 14.0, 13.3, OCH_3 , 14.25 $\text{C}_{17}\text{H}_{12}\text{O}_6\text{N}_4(\text{OCH}_3)_2$ requires C, 53.02, H, 4.21, N, 13.0, OCH_3 , 14.4%) The substance is insoluble in aqueous NaHCO_3 , Na_2CO_3 , cold NaOH or dilute ammonia, but dissolves slowly in NaOH on heating producing a brown solution. The substance therefore, does not contain a free carboxyl group although it may have a lactone group.

Oxidation of the aldehyde, $\text{C}_{12}\text{H}_{14}\text{O}_5$, with alkaline iodine Formation of a monobasic acid monolactone, $\text{C}_{12}\text{H}_{14}\text{O}_6$. The aldehyde $\text{C}_{12}\text{H}_{14}\text{O}_5$ (1.0 g), was dissolved in methanol (5 ml) by warming cooled and treated with 0.1N iodine (200 ml.)

with vigorous shaking. 0.1N NaOH (250 ml) was added, and the whole was well shaken until all the oil disappeared. The liquid became colourless. After 30 min $N \text{H}_2\text{SO}_4$ (25 ml) was added and the liberated I_2 was titrated with 0.5N $\text{Na}_2\text{S}_2\text{O}_3$. The colourless mixture was made acid to Congo red and extracted four times with an equal volume of ether. The ethereal extract was shaken with aqueous NaHCO_3 (20 ml), and the bicarbonate solution on acidification gave colourless crystals, 0.24 g, m.p. 147°, which on recrystallization from ether light petroleum gave colourless feathery needles, m.p. 151–152° (Found C, 58.72, 58.60, H, 5.14, 5.20, OCH_3 , 23.4 $\text{C}_{11}\text{H}_{12}\text{O}_4(\text{OCH}_3)_2$ requires C, 58.64, H, 5.30, OCH_3 , 23.3%) 0.2023 g of the acid was titrated in the cold to phenolphthalein with 0.1N NaOH requiring 7.52 ml. corresponding to an equivalent of 269 $\text{C}_{12}\text{H}_{14}\text{O}_6$ titrating as a monobasic acid requires 266. A further 20.00 ml. of 0.1N NaOH was added and the solution was boiled under reflux, with exclusion of CO_2 , for 2.5 hr. A back titration used 12.48 ml. of 0.1N H_2SO_4 . Thus the additional acid groups unmasked were equivalent to 7.52 ml. 0.1N alkali. A second acid group has thus been revealed by alkaline hydrolysis, the substance behaves as a mono acid monolactone.

In a second oxidation experiment, in which a proportionately much larger amount of methanol was used ($\text{C}_{12}\text{H}_{14}\text{O}_5$ aldehyde, 0.25 g, methanol, 7 ml, 0.1N I_2 , 60 ml., 0.1N NaOH , 75 ml), the products extracted by ether, after acidification, were separated into acidic and neutral fractions by means of NaHCO_3 . The acidic fraction yielded the acid $\text{C}_{12}\text{H}_{14}\text{O}_6$, m.p. 151–152°, referred to above. The neutral fraction yielded, after several recrystallizations from ether, large colourless prisms, m.p. 94–95° (Found C, 59.88, H, 5.92, OCH_3 , 33.1, 32.9 $\text{C}_{11}\text{H}_{12}\text{O}_5(\text{OCH}_3)_2$ requires C, 59.98, H, 5.75, OCH_3 , 33.2%) This product thus appears to be the methyl ester of the acid lactone $\text{C}_{12}\text{H}_{14}\text{O}_6$, m.p. 151–152°. Its formation must be due to the use of methanol to dissolve the aldehyde prior to oxidation.

Oxidation of the monobasic acid monolactone, $\text{C}_{12}\text{H}_{14}\text{O}_6$, with KMnO_4 Formation of a tribasic acid, $\text{C}_{12}\text{H}_{12}\text{O}_7$. The monobasic acid monolactone $\text{C}_{12}\text{H}_{14}\text{O}_6$ (0.5 g) was boiled for 1.5 hr under reflux with $N \text{NaOH}$ (10 ml.) and water (50 ml.) to open the lactone ring. When cold the solution was treated with a cold solution of KMnO_4 (0.4 g, equivalent to 2 atoms O) in water (50 ml). The mixture was left for 3 days at room temperature. The precipitated MnO_2 was removed by filtration. The pale green filtrate was acidified to Congo red with H_2SO_4 and extracted with ether. On removal of the solvent a solid residue was obtained, 0.56 g, which proved to be a mixture of substances. It was fractionated by solution in water, neutralization with NaOH , and precipitation with aqueous neutral lead acetate (10 ml of 0.5N). The white precipitate thus obtained was collected, washed with water, acidified with H_2SO_4 and extracted with ether. On removal of the ether the dry product (0.19 g) obtained was recrystallized from ether light petroleum, giving 0.10 g of colourless slender prisms m.p. 161–162°, raised to 162.5° by sublimation *in vacuo* (Found C 55.80, H, 4.37, OCH_3 , 23.0, neutralization equivalent in cold 96.8 $\text{C}_{11}\text{H}_6\text{O}_5(\text{OCH}_3)_2$ requires C, 55.71, H, 4.32, OCH_3 , 22.1%, equivalent titrating as a tribasic acid, 93.4.)

III Action of ozone on mycophenolic acid monomethyl ether methyl ester Mycophenolic acid monomethyl ether methyl ester (5.6 g), m.p. 58° (Clutterbuck & Raistrick, 1933, p. 657), in chloroform (250 ml) was ozonized for 10 hr.

Water fraction This fraction contained very little material giving a precipitate with Brady's reagent. Its ether extract

was split as in sections AI and AII, by extraction with aqueous NaHCO_3 , into (a) neutral fraction, 0.2 g oil, (b) acid fraction, 0.15 g oil. Neither fraction was examined further

Chloroform fraction The acid fraction, 0.12 g of crystals, was not further examined. The neutral fraction, 5.4 g of oil, was distilled *in vacuo* and gave the following fractions

Fraction 1, b.p. 45–55°/1 mm, 0.86 g, mobile liquid

Fraction 2, b.p. 190°/1 mm, 0.96 g, viscous oil, depositing crystals

Fraction 3, b.p. 195°/1 mm, 0.96 g, viscous oil, depositing crystals

Fraction 4, undistilled residue. Some decomposition. Not further examined.

Fraction 1 0.1 g treated with Brady's reagent (50 ml.) gave crystalline orange needles, 0.16 g, m.p. 137°. It recrystallized readily from ethanol in bright-orange flattened needles, m.p. 137–138°, which did not depress the melting point of the mono 2,4-dinitrophenylhydrazone of synthetic methyllevulic acid, m.p. 139° (Found C, 46.56, 46.59, H, 4.65, 4.45, N, 18.3, OCH_3 , 10.1 $\text{C}_{11}\text{H}_{11}\text{O}_5\text{N}_4(\text{OCH}_3)$ requires C, 46.44, H, 4.55, N, 18.05, OCH_3 , 10.0%)

Fractions 2 and 3 were combined and pressed on porous tile, giving 0.87 g of colourless crystals, which after recrystallization from light petroleum, b.p. 40–60°, or water gave a strong Schiff reaction, and melted at 112° alone or in admixture with the aldehyde $\text{C}_{13}\text{H}_{14}\text{O}_5$ obtained by ozonization of mycophenolic acid monomethyl ether (see section AII) (Found C, 62.15, 62.33, H, 5.75, 5.81, OCH_3 , 24.7, neutralization equivalent in hot solution, 240 $\text{C}_{11}\text{H}_9\text{O}_5(\text{OCH}_3)_2$ requires C, 62.40, H, 5.64, OCH_3 , 24.8%, neutralization equivalent, titrating as a monobasic acid, 250) It also gave the same 2,4-dinitrophenylhydrazone, m.p. and mixed m.p. 214–215° (Found C, 53.10, H, 4.31, OCH_3 , 14.2 $\text{C}_{17}\text{H}_{15}\text{O}_6\text{N}_4(\text{OCH}_3)_2$ requires C, 53.02, H, 4.21, OCH_3 , 14.4%)

B Acetylation of the aldehyde, $\text{C}_{13}\text{H}_{14}\text{O}_5$ Formation of an enol mono-acetate

The aldehyde, m.p. 112° (0.10 g), was heated with anhydrous sodium acetate (0.2 g) and acetic anhydride (0.4 ml) until the mixture began to boil and was then maintained at 140° for 0.5 hr. There was only a slight discoloration. The mixture was poured into water and stirred. A white flocculent precipitate (0.08 g, m.p. 130–133°) separated. Recrystallization from water and light petroleum, b.p. 80–100°, gave colourless needles, m.p. 140° (Found C, 61.30, H, 5.70, CH_3CO , 17.0, $\text{CH}_3(\text{C})$, 10.6 $\text{C}_{11}\text{H}_7\text{O}_5(\text{CH}_3\text{CO})(\text{CH}_3)_2$ requires C, 61.65, H, 5.52, CH_3CO , 14.7, $2\text{CH}_3(\text{C})$, 10.3%) The product gave no reaction with 2,4-dinitrophenylhydrazine (Brady's reagent) or with Schiff's reagent. It is thus clearly an enol mono acetate of the $\text{C}_{13}\text{H}_{14}\text{O}_5$ aldehyde

Ozonization of the enol acetate. Formation of an aldehyde, $\text{C}_{13}\text{H}_{14}\text{O}_5$ A solution of the enol acetate (0.2 g) in chloroform (20 ml.) was ozonized for 45 min. After treatment with hot water the chloroform layer was separated, washed with a little aqueous NaHCO_3 , dried with Na_2SO_4 and evaporated. The oil (0.12 g) remaining partly crystallized *in vacuo*. The whole was crystallized from light petroleum, b.p. 80–100°, and gave 25 mg of colourless needles, m.p. 118°, which

were sublimed in a high vacuum giving a colourless crystalline sublimate m.p. 125–126° (Found C, 60.36, H, 4.92 $\text{C}_{12}\text{H}_{12}\text{O}_5$ requires C, 61.01, H, 5.12%) The product gave a precipitate with Brady's reagent and a positive reaction with Schiff's reagent

The residual oil (90 mg), recovered from the light petroleum mother liquors, was treated with ethanol (2 ml.) and Brady's reagent (60 ml.) The crude red-orange 2,4-dinitrophenylhydrazone (115 mg) was recrystallized from benzene, and gave 60 mg of red brown glistening needles containing one molecule of benzene of crystallization. On heating there was slight softening at 212–214°, with fairly sharp melting at 228–230° with slight effervescence (Found, on air dried crystals C, 58.60, H, 4.51, N, 11.8, loss in high vacuum at 100–110°, 19.7 $\text{C}_{18}\text{H}_{16}\text{O}_5\text{N}_4\text{C}_6\text{H}_6$ requires C, 58.30, H, 4.49, N, 11.3, loss 15.8% Found, on crystals dried to constant weight C, 51.76, H, 3.96, N, 13.3 $\text{C}_{18}\text{H}_{16}\text{O}_5\text{N}_4$, i.e. dinitrophenylhydrazone of $\text{C}_{12}\text{H}_{12}\text{O}_5$, requires C, 51.94, H, 3.88, N, 13.45%)

C Colour reactions of mycophenolic and normycophenolic acids with 2,6-dichloroquinone chloroimide

A small amount of each acid was dissolved in 2–3 ml of sodium borate buffer, pH 9.2. To each solution was added two drops of a suspension of 2,6-dichloroquinone chloroimide in water. Mycophenolic acid gave a very faint dirty yellow colour, unchanged after 40 min. Normycophenolic acid rapidly gave a colour, blue with a tinge of green, inclining after 40 min to purple. A control experiment using buffer solution and quinone alone gave a very faint pink unchanged after 40 min.

D Carbon methyl group estimations

(a) On the aldehyde $\text{C}_{13}\text{H}_{14}\text{O}_5$. Found $\text{CH}_3(\text{C})$, 4.75% $\text{C}_{13}\text{H}_{14}\text{O}_5$ with one $\text{CH}_3(\text{C})$ group requires 6.00%. This corresponds to 79% of the theoretical for one $\text{CH}_3(\text{C})$ group

(b) On mycophenolic acid. Found $\text{CH}_3(\text{C})$, 6.57% $\text{C}_{13}\text{H}_{14}\text{O}_5$ with two $\text{CH}_3(\text{C})$ groups requires 9.38%. This corresponds to 140% of the theoretical for one $\text{CH}_3(\text{C})$ group and to 70% of the theoretical for two $\text{CH}_3(\text{C})$ groups

SUMMARY

1 Further degradative experiments have been carried out on mycophenolic acid, a metabolic product of species and strains in the *Penicillium brevicompactum* Dierckx series. These experiments consist mainly of a study of the products formed by the ozonolysis of mycophenolic acid and some of its methylated derivatives and breakdown products

2 They lead to two possible structural formulae for mycophenolic acid, one being more probable than, and preferred to, the other. Mycophenolic acid may be described as 6-hydroxy-4-methoxy-3(or 5)-methyl-5(or 3)-(5' carboxy-3' methylpent-2' enyl)phthalide

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The Oxidation of Diiodotyrosine Derivatives

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It is now well established that when duodotyrosine is incubated under mildly alkaline conditions, thyroxine is formed (von Mutzenbecher, 1939, Block, 1940, Johnson & Tewkesbury, 1942, Harington & Pitt Rivers, 1945), under the most favourable conditions, however, the yields of thyroxine are small, and a large proportion of the duodotyrosine is lost, giving rise to tarry by products, inorganic iodide and ammonia. So far, no compound has been isolated after such an incubation which would indicate through what intermediate step thyroxine is synthesized, nor has the side chain, which is removed from one molecule of duodotyrosine during the coupling of two molecules to give thyroxine, or a recognizable derivative thereof, been isolated, although Johnson & Tewkesbury (1942) claim to have detected pyruvic acid in the reaction mixture when thyroxine is formed under these conditions.

It seemed possible that further investigation of the products of alkaline oxidation of duodotyrosine might reveal some compound which would give a clue to the mechanism of the reaction. It had been shown by Harington & Pitt Rivers (1945) that the pH at which duodotyrosine is incubated exerts a marked effect both on the amount of thyroxine formed and on the extent to which duodotyrosine itself is oxidized. At a high pH (13 or 14) no thyroxine was obtained by these authors, but instead another crystalline iodine containing compound was isolated, which was not characterized at the time. This compound has now been re-examined and has been identified as 3,5-diiodo-4-hydroxybenzaldehyde. Further examination of the products of incubation of duodotyrosine at pH 10 has shown that at this pH the aldehyde is formed at the same

time as thyroxine, at lower pH (7.8–8.8) none of the aldehyde is obtained, while the yields of thyroxine are very small.

The simultaneous formation of 3,5-diiodo-4-hydroxybenzaldehyde and thyroxine from duodotyrosine led first to the hypothesis that the aldehyde might be an intermediate in thyroxine synthesis, and attempts were made to demonstrate this by incubating duodotyrosine in the presence of the aldehyde, in the hope that increased yields of thyroxine might result. These attempts have proved abortive, and in all the experiments done the yield of thyroxine was unchanged and the aldehyde was recovered at the end of the reaction. Further, Dakin's (1909) observation that 3,5-diiodo-4-hydroxybenzaldehyde is unaffected by alkaline hydrogen peroxide, while the dichloro and dibromo analogues give more or less good yields of the corresponding quinols, has been confirmed. Only under the most drastic conditions may the diiodo aldehyde be oxidized, and the product of oxidation is 2,4,6-triodophenol. These facts have, therefore, led to the abandonment of the hypothesis that the aldehyde is an intermediate in thyroxine synthesis, and it is now thought that its formation from duodotyrosine is due to a different mechanism from that which leads to thyroxine.

The mode of formation of 3,5-diiodo-4-hydroxybenzaldehyde from duodotyrosine is itself of some interest. It has been found that, when the aldehyde is obtained by incubating duodotyrosine at pH 14, an amount of oxalic acid can be isolated as the calcium salt from the reaction mixture which is molecularly equivalent to the amount of aldehyde isolated, this leads to the suggestion that the

reaction entails, first, oxidation on the β carbon atom of the duodotyrosine side chain. Further, when duodotyrosine is incubated at pH 10, with the simultaneous formation of thyroxine and the aldehyde, oxalic acid is again obtained. In this instance the amount of oxalic acid appeared, in two experiments, to be molecularly equivalent to the sum of thyroxine and diodo *p* hydroxybenzaldehyde isolated. This observation suggested that the aldehyde and thyroxine might be obtained by a mechanism whose initial steps were similar, but which, under the influence of pH or other factors (Pitt-Rivers, 1947), led to different end products. Even if we suppose that the apparent equivalence, mentioned above, of the oxalic acid formed with the sum of aldehyde and thyroxine produced at pH 10 is a genuine phenomenon, the observation is of little value in relation to the essential question of the form in which the side chain of one of the reacting molecules of duodotyrosine is eliminated, oxalic acid is the common end product of oxidation of many 2- and 3 carbon aliphatic compounds, and in the present experiments its appearance could be explained equally well by the stepwise degradation of the side chain involving primary scission of the two terminal carbon atoms, or by secondary oxidation following elimination of the whole side chain as a 3 carbon fragment.

From what has already been said, it appears that the formation of 3,5 diodo 4 hydroxybenzaldehyde from duodotyrosine depends upon a certain degree of alkalinity, and in this connexion the formation of acid-insoluble iodine containing compounds other than thyroxine during the preparation of artificial thyroproteins is called to mind.

During the preparation of such proteins by the method of Ludwig & von Mutzenbecher (1939), later developed by other workers (Reineke & Turner, 1942; Pitt-Rivers & Randall, 1945), the initial pH of the protein solution in bicarbonate buffer is about 8 and may rise to 9 during the iodination, it may also be remembered that Deanesly & Parkes (1945) showed that the method of assay as used for thyroid powder (determination of the acid insoluble iodine content) cannot be applied to the artificial thyroproteins, since they contain large amounts of acid-insoluble iodine containing products which are biologically inactive. The inactivity of 3,5 diodo-4 hydroxybenzaldehyde as measured by the tadpole metamorphosis test, together with the effect of alkaline conditions on aldehyde production from duodotyrosine, suggested that this aldehyde might be isolated from the products of baryta hydrolysis of such a thyroprotein. This has proved to be so, and in one instance the amounts of aldehyde and thyroxine isolated were in the ratio of 10:1. No aldehyde has been isolated following baryta hydrolysis of desiccated thyroid powder.

The instability of duodotyrosine itself is so great that it does not permit readily of its use in determining in what form the 3 carbon side chain is eliminated during thyroxine synthesis. In a further attempt to elucidate this point, a method of stabilizing the side chain has therefore been sought, and it was thought probable that acetylation of the amino group might achieve this result. Preliminary experiments on the alkaline incubation of *N* acetyl L duodotyrosine in solutions with a pH range of 7-8.5, followed by alkaline hydrolysis and butanol fractionation of the products, led to the isolation of thyroxine in small yields. During these incubations, however, it was noticed that a crystalline salt separated. This proved to be the sodium salt of *N*-acetyl L thyroxine, and from it the free acid could be obtained in yields of about 25%, calculated on the amount of unchanged starting material recoverable at the end of the experiments. *N*-Acetyl L duodotyrosine has been titrated potentiometrically, and the pH optimum for acetyl thyroxine formation has been determined. Acid hydrolysis of acetyl thyroxine in a mixture of acetic acid and concentrated hydrochloric acid yielded L thyroxine with $[\alpha]_D^{21} - 5.42^\circ$ in 3% solution in a mixture of two parts ethanol and one part *N* NaOH.

In the thyroid gland itself, thyroxine must, in part, be formed from duodotyrosine molecules, which are joined through both their amino and carboxyl groups in peptide linkage, it appeared, therefore, to be of interest to discover whether a duodotyrosine derivative, in which both these groups are blocked, would lead *in vitro* to the formation of a corresponding thyroxine derivative, and *N*-acetyl-DL duodotyrosylglutamic acid was prepared for this purpose. Incubation of the acetyl peptide at pH 7.2 in dilute sodium hydroxide solution led to no insoluble salt of a thyroxine derivative, but alkaline hydrolysis of the reaction mixture did yield a small amount of thyroxine. If the incubation of the acetyl peptide were carried out at pH 7.2 in baryta, however, a partly crystalline barium salt separated which, after decomposition with acid, yielded *N* acetyl-DL-thyroxyl L glutamic acid. This thyroxine derivative has been obtained in yields up to 36% net, and conversion of the duodotyrosine derivative to the thyroxine derivative is particularly good in this instance, gross yields being of the order of 20%. Acid hydrolysis of acetylthyroxylglutamic acid led to the isolation of thyroxine and glutamic acid.

The good yields of acetylthyroxine and the acetylthyroxine peptide obtained in these experiments, together with the relative freedom of the reaction mixtures from tarry products, have led to the hope that the duodotyrosine side chain which is detached might be isolated, either as an acetamido compound or, after hydrolysis, as an amino acid. After 2 or 3 weeks' incubation of acetyl duodotyrosine in

baryta and removal of acetylthroxine and of as much as possible of the starting material, the residue was hydrolyzed with sulphuric acid and freed from diiodotyrosine, iodide and sulphate. The residual solution contained an amino acid (positive ninhydrin reaction) and chromatographic examination, kindly carried out by Dr R. L. M. Synge, revealed in two separate experiments the presence of small amounts of alanine. Further experiments are being carried out in an effort to isolate this amino acid, and to discover whether this represents the diiodotyrosine side chain which is split off. Serine and glycine have been looked for, but Dr Synge has reported that there is no evidence of their presence.

EXPERIMENTAL

Isolation and identification of 3,5-diiodo-4-hydroxybenzaldehyde from L-diiodotyrosine after oxidation at pH 14 with H_2O_2 (hot)

L-Diiodotyrosine ($2H_2O$ (28.2 g)) was dissolved in a mixture of 12 ml 10N NaOH and 100 ml 2N NaOH, and heated on a boiling water bath. 20 vol H_2O_2 (60 ml) were added, and after the vigorous reaction had subsided the solution was cooled and extracted with an equal volume of *n*-butanol, these operations were repeated three times. The pooled butanol extracts were washed once with half their volume of 2N NaOH and concentrated to dryness. The crystalline residue was dissolved in water (200 ml) and acidified at the boiling point with 14N H_2SO_4 . A white crystalline precipitate immediately appeared which was collected, washed with water and dried *in vacuo* over P_2O_5 . The yield was 1.11 g. After recrystallization from benzene 0.71 g of the product was obtained (prisms), and had m.p. 194–196° (decomp.) not depressed in admixture with authentic 3,5-diiodo-4-hydroxybenzaldehyde (Found C 22.6, H 1.2, I 68.0. Calc. for $C_7H_3O_2I_2$, C 22.5, H 1.07, I 67.9%).

Reduction of the above compound 0.42 g was dissolved in 7 ml 5N NaOH and 20 ml water, and hydrogenated in the presence of 0.25 g palladized strontium carbonate. At the end of 2 hr the reduction was complete and 40 ml of hydrogen had been taken up. The reaction mixture was filtered, treated with 1.4 ml 5N H_2SO_4 and concentrated to dryness. The residue was crystallized from the minimum amount of hot water. The aqueous mother liquor was again concentrated to dryness and crystallized from water, 90 mg product were obtained with m.p. 111–112°, and were recrystallized from water in needles with m.p. 114–115°. The addition of *p*-hydroxybenzaldehyde did not depress the melting point.

The product formed a semicarbazone with m.p. 218–220° which, with *p*-hydroxybenzaldehyde semicarbazone had m.p. 219–220° (Found N, 23.0. Calc. for $C_7H_5O_2N$, N 23.5%).

Isolation of 3,5-diiodo-4-hydroxybenzaldehyde and oxalic acid after incubation of L-diiodotyrosine for 14 days at pH 14

Diiodotyrosine ($2H_2O$ (46.9 g)) was dissolved in a mixture of 300 ml 5N NaOH and 20 ml 10N NaOH, and incubated for 14 days after which the solution was extracted once with

an equal volume of butanol. After washing the butanol extract with about 50 ml 5N NaOH it was concentrated to dryness. It yielded 0.308 g crystalline product with m.p. 184–190° (decomp.) with previous darkening. The product was then extracted repeatedly with hot 20% sodium metabisulphite solution, decomposition of which, with mineral acid, gave 0.235 g of 3,5-diiodo-4-hydroxybenzaldehyde, m.p. 198–200° (decomp.), the mixed melting point with the authentic aldehyde was not depressed. The aqueous fraction was freed from butanol by a short distillation under diminished pressure, and the pH was brought up to 4.3 by addition of conc. HCl. The diiodotyrosine which separated was allowed to crystallize at 4° during 4–5 hr and was then collected, washed with a little water and air-dried. Recovered diiodotyrosine, 44.8 g.

The filtrate was brought to pH 5.5 with NaOH and treated with 2 ml 5M $CaCl_2$ solution. After a short time calcium oxalate began to separate, and crystallization was completed by leaving at room temperature for 15 hr. The precipitate was collected on the centrifuge, washed with water and determined by titration with 0.1N $KMnO_4$. Oxalic acid found 47 mg. The volume of the solution from which the calcium oxalate had separated was 850 ml. Calcium oxalate is soluble in water at 25° to the extent of 0.7 mg/100 ml, so a correction for this was made bringing the oxalic acid to 51.2 mg (0.57 mmol.). The amount of diiodo-*p*-hydroxybenzaldehyde found (0.235 g) was 0.63 mmol.

Formation of oxalic acid during incubation of diiodotyrosine at pH 10

(a) Diiodotyrosine ($2H_2O$ (46.9 g)) was incubated at pH 10 as described by Harrington & Pitt-Rivers (1945). The butanol soluble fraction yielded acid insoluble material weighing 1.08 g. This was extracted three times with about 25 ml ether, then twice with 20 ml ethyl acetate and again with ether. The ether insoluble portion, on dissolving in boiling 0.1N Na_2CO_3 and working up as usual gave 0.12 g thyroxine.

The ether ethyl acetate soluble portion after removal of solvent weighed 0.82 g. Extraction with hot 20% sodium metabisulphite solution, until no further material would dissolve, gave in all 0.46 g diiodo-*p*-hydroxybenzaldehyde, m.p. 196–198° (decomp.).

From the aqueous fraction, 41.68 g diiodotyrosine were recovered. 5M $CaCl_2$ solution was added to the filtrate and the precipitated oxalate was collected, redissolved in acid, filtered from a small amount of brownish material, reprecipitated, collected by centrifuging and washed with water. The calcium oxalate was then dissolved in 5N H_2SO_4 and the solution was divided into halves. A determination of oxalate by permanganate titration was made on the one half while the other half was extracted many times with ether. The ether extract on evaporating to dryness gave crystals which on recrystallization from water had m.p. 100–101°, not depressed in admixture with oxalic acid dihydrate. The permanganate titration showed that there was present 0.190 g calcium oxalate (1.46 mmol). Thyroxine isolated was 0.12 g (0.155 mmol). diiodo-*p*-hydroxybenzaldehyde was 0.46 g (1.23 mmol).

(b) A repetition of the above experiment on 35.2 g diiodotyrosine ($2H_2O$) with 7 days incubation gave thyroxine 39 mg (0.05 mmol.), diiodo-*p*-hydroxybenzaldehyde 0.28 g (0.75 mmol.) and oxalic acid 0.78 g (0.87 mmol.).

Incubation of diiodotyrosine at pH 7.8

This was done in exactly the manner described by Harrington & Pitt-Rivers (1945). The butanol extract gave an acid insoluble crude product weighing 69 mg. After extraction with ether, this fraction weighed 15 mg, from which 8 mg pure thyroxine were obtained. The ether extract on being concentrated to dryness and treated with metabisulphite gave no aldehyde which could be weighed, although a trace of material (less than 1 mg) was seen in the acidified metabisulphite solution which crystallized in the characteristic prisms of diiodo-*p* hydroxybenzaldehyde. The recovered diiodotyrosine weighed 8.09 g, which gave a net yield of thyroxine of 0.74%.

Products obtained from the hot peroxide oxidation of diiodotyrosine

L-Diiodotyrosine (23.5 g) was dissolved in 0.96N NaOH (99 ml.) and heated on a boiling water bath. Butanol (150 ml) was added, and then 20 vol H_2O_2 (150 ml) was added in portions with continuous vigorous shaking. When there was no further effervescence the reaction mixture was cooled and the butanol layer separated. This was repeated twice, using 250 ml. butanol for the subsequent extractions. The pH was kept just alkaline to phenolphthalein throughout the reaction by small additions of N NaOH, each butanol extract was washed with 100 ml N NaOH and the pooled washings were re-extracted with 250 ml butanol.

The butanol soluble fraction contained 3.18 g of acid insoluble material. This was extracted seventeen times by grinding with ether. The ether insoluble portion gave 0.25 g thyroxine after purification through the sodium salt.

The ether extract was concentrated to dryness. Repeated extraction with hot 20% sodium metabisulphite gave 0.29 g of a crystalline compound, m.p. 198–199° (decomp.), mixed melting point with 3,5-diiodo-4-hydroxybenzaldehyde, 200–201° (decomp.). The residue after bisulphite extraction was steam distilled, the steam volatile material weighed 0.144 g and had m.p. 154–156° not depressed by mixing with 2,4,6-triodophenol (Found I, 80.5 and 81% on duplicate analysis. Calc. for $\text{C}_9\text{H}_5\text{OI}_3$, I, 80.7%).

The aqueous residue after butanol extraction was added to the butanol washings and acidified at the boiling point. A dark brown tar separated which solidified on cooling, it was ground with water, collected and dried, and weighed 7.45 g. It contained no steam volatile material.

The tar (5 g) was dissolved in N KOH (165 ml) and reduced with hydrogen in the presence of palladized strontium carbonate (15 g). At the end of the reduction the solution was filtered from catalyst, concentrated to a low volume and acidified with 5N-HCl, 0.75 g of insoluble material was precipitated and was extracted several times with ether. The ether extract on evaporating to dryness gave a solid residue which, on crystallization from water, yielded 35 mg of a crystalline compound with m.p. 208°. The melting point of this compound was not depressed by mixing with *p* hydroxybenzoic acid with m.p. 211–213°.

Of the original unreduced tar, 0.4 g was extracted with warm saturated sodium acetate solution. The extract was diluted with two parts of water and acidified at the boiling point with conc. HCl. Sheaves of needles separated on cooling and weighed 30 mg. They had m.p. about 220°

(decomp.) with loss of iodine above 200°. Mixed melting point with 3,5-diiodo-4-hydroxybenzoic acid was not depressed.

*Preparation of biologically active iodinated casein and isolation therefrom of thyroxine and diiodo-*p* hydroxybenzaldehyde*

Casein (Kahlbaum nach Hammarsten) (50 g) was suspended in water (2 l) and dissolved with the aid of 2N NaOH. NaHCO_3 (15 g) was added and the pH was brought back to 8 by addition of 2N HCl, 19 ml of a 4.16N I_2 solution were added over a period of 2.5 hr while the solution was mechanically stirred at 40°. The temperature was then raised to between 60 and 70° and maintained there for 5 hr, at the end of which the pH was 8.9 (British Drug Houses Ltd capillator).

The iodinated casein was then precipitated by bringing the reaction mixture to pH 4 by addition of HCl. The separated protein was redissolved in the same volume of water by addition of alkali, reprecipitated with acetic acid, collected, washed with about 450 ml N/700 acetic acid and air dried. After some accidental loss the yield was 38 g.

Of this preparation 35 g were boiled for 20 hr under reflux with 500 ml water and 200 g recrystallized Ba(OH). The hydrolysate was filtered hot, and the decomposed barium salt was combined with the acid insoluble material in the filtrate. This product weighed 0.82 g, it was extracted twice with ether, once with ethyl acetate and then three times again with ether. The residue weighed 0.78 g, and yielded in all 108 mg pure thyroxine, from which the thyroxine content of the protein was 0.31%. The pooled ether and ethyl acetate extracts were combined and evaporated to dryness, and the crystalline residue was purified by extraction with hot 10% sodium metabisulphite. The crystalline compound obtained by decomposition of this extract weighed 15 mg and had m.p. 197–198° (decomp.). The mixed melting point with authentic 3,5-diiodo-4-hydroxybenzaldehyde was not depressed.

Isolation of 3,5-diiodo-4-hydroxybenzaldehyde from iodinated proteins with low biological activity

The method was as follows: 50–100 g protein were boiled with 20 parts 2N NaOH for 18 hr, and then cooled, strained through glass wool if necessary, and extracted once with an equal volume of butanol. The butanol fraction was worked up as described in the previous hydrolysis.

Iodinated casein (PB11) DT/5/829 contained 8.9% total I and 3.3% acid insoluble I (see Deanesly & Parkes, 1945), 60 g yielded 23 mg of pure thyroxine (giving 0.038% thyroxine) and 45 mg (0.075%) pure diiodo-*p* hydroxybenzaldehyde.

Iodinated casein (PB6) DT/5/823 contained 7.4% total I and 2.3% acid insoluble I (see Deanesly & Parkes, 1945). Hydrolysis of 50 g of this preparation for 7 hr with 4N NaOH, followed by butanol extraction, yielded no thyroxine and only 5–6 mg pure aldehyde.

Hydrolysis of a mixture of iodinated caseins PB6 and PB11 with fractionation of acid insoluble I

PB6 (50 g) + PB11 (15 g) were boiled under reflux for 16.5 hr with 1 l 2N NaOH. After cooling, the hydrolysate was extracted with an equal volume of butanol. This gave

an acid insoluble precipitate of 1.05 g containing 27.8% I, and after purification through the usual processes gave thyroxine (3.0 mg) and 3.5 diodo-4-hydroxybenzaldehyde (30 mg). The aqueous fraction contained 6.77 g acid insoluble material containing 5.51% I, from which no crystalline compound could be obtained.

Under these conditions of hydrolysis, it therefore appears that (i) of the acid insoluble iodine found (665 mg) 56% is not soluble in butanol, and (ii) that of the butanol soluble acid insoluble iodine, less than 1% could be accounted for as thyroxine, while 7% was accounted for as diodo-*p*-hydroxybenzaldehyde, 92% of this butanol soluble iodine was not characterized.

Hydrolysis of desiccated thyroid powder B8

Defatted thyroid powder (200 g) was boiled under reflux with 21 N NaOH for 17 hr, and fractionated into butanol as described above. The acid insoluble residue from the butanol fraction weighed 5.6 g. This fraction was composed largely of fat and was largely soluble in ether. The ether soluble material was boiled three times with 20% sodium metabisulphite solution, and each extract was cooled before filtering (since the bisulphite compound of 3.5 diodo-4-hydroxybenzaldehyde is soluble in cold water, this procedure did not risk the loss of aldehyde). Decomposition of the bisulphite solution with mineral acid led to no insoluble material, so that no aldehyde appeared to be present. Since it was possible that the aldehyde might not be extractable from the fat with any ease, about 1 mg authentic aldehyde was added to it; one bisulphite extract easily removed it.

Experiments on the incubation products of *N*-acetyl L-diiodotyrosine

N-Acetyl L-diiodotyrosine. This compound has already been prepared by Myers (1932), but the method is not suitable for large amounts. Moreover, the compound is unstable and tends to become coloured during recrystallization. The following method gives a product which may be used for incubation experiments without further crystallization. *ON* Diacetyl L-diiodotyrosine (Myers, 1932) (25.85 g) was dissolved in 150 ml cold \sim NaOH. After 10 min. at room temperature, the solution was filtered, diluted to 600 ml with water, warmed to 75° and acidified by addition of 170 ml \sim HCl with stirring, the product began to crystallize immediately and the solution was then cooled rapidly to 0°. After 2 hr in the ice chest the crystals were collected, washed with water and dried *in vacuo* over P_2O_5 , the yield was 22.5 g, *m.p.* 125°, $[\alpha]_D^{25} + 49.7^\circ$ in 2% solution in \sim NaOH (Found C 27.3, H, 2.6 I 52.0. Calc for $C_{11}H_{11}O_4N_2I_2 \cdot \frac{1}{2}H_2O$ C, 27.3, H, 2.5 I, 52.5%).

Apparent dissociation constant of the diiodophenolic group of *N*-acetyl L-diiodotyrosine. The monosodium salt of *N*-acetyl L-diiodotyrosine was titrated potentiometrically in 0.05N solution at 23 and 37°. At 23° the apparent *pH* was 6.95, at 37° it was about 6.75, but at the higher temperature the compound was not stable and the *pH* values calculated are not as constant as those obtained from the curve at 23°. This result is in fairly close agreement with the values calculated by Winick & Schmidt (1933) for the apparent dissociation constant of the diiodophenolic group of diiodotyrosine at different temperatures. These authors found that the apparent *pH* calculated from solubility determinations was 6.53 at 0°, 6.45 at 25° and 6.45 at 40°.

Formation of thyroxine during aerobic incubation of *N*-acetyl L-diiodotyrosine. *N*-Acetyl L-diiodotyrosine (9.5 g) was suspended in 100 ml water, and dissolved by adding 37 ml \sim NaOH, the initial *pH* was 7.6. After 14 days at 37° the reaction mixture was made 4N with respect to NaOH and boiled under reflux for 1.5 hr. The cooled solution was diluted with an equal volume of water and extracted twice with an equal volume of butanol. Purification of the butanol soluble fraction in the usual manner (Harrington & Pitt Rivers, 1945) yielded 144 mg pure thyroxine, 5.25 g diiodotyrosine was recovered from the aqueous fraction. The net yield of thyroxine was therefore 4.7%.

Effect of *pH* on thyroxine formation. *N*-Acetyl L-diiodotyrosine was dissolved in water and different amounts of alkali, the initial *pH* of each solution being calculated from the titration curve. After 14 days at 37°, the thyroxine formed and residual diiodotyrosine were separated as described above. The results are given in Table 1.

Table 1 Formation of thyroxine from *N*-acetyl L-diiodotyrosine incubated at different *pH*

Acetyl diiodo tyrosine incubated (g)	<i>pH</i>	Thyroxine isolated (mg)	Diiodo-tyrosine recovered (g)	Net yield of thyroxine (%)
7.13	7.15	70	2.67	2.0
6.5	7.25	122	2.73	4.3
9.5	7.45	162	5.58	5.9
9.5	7.70	144	5.25	4.7
9.5	8.0	84	0.96	4.2
9.5	8.35	74	6.72	3.4

Isolation of *N*-acetyl L-thyroxine from the aerobic incubation products of *N*-acetyl L-diiodotyrosine. *N*-Acetyl L-diiodotyrosine (9.5 g) was dissolved in 100 ml 0.05N borate buffer at *pH* 7.6 and 17.8 ml \sim NaOH, the initial *pH* was 7.6. After 2 days at 37° a crystalline sodium salt began to separate, and this slowly increased in amount. After 12 days' incubation, the sodium salt was separated on the centrifuge and washed with a little water. Decomposition of this salt with dilute HCl led to the isolation of a crystalline compound weighing 290 mg. Recrystallization by the method of Ashley & Harrington (1929) gave a product which crystallized in long thin prisms and which had *m.p.* 232–234° (decomp), $[\alpha]_D^{25} + 25.7$ in 4% solution in a mixture of equal volumes of ethanol and \sim NaOH (Found I, 62.0. Calc for *N*-acetyl thyroxine, $C_{17}H_{15}O_5N_2I_2$, I, 62.0%). From the mother liquor 8.25 g *N*-acetyl L-diiodotyrosine was recovered, the net yield of the acetylthyroxine was therefore 27%. Incubations of *N*-acetyl L-diiodotyrosine in baryta solution at *pH* 7.6 led to the separation of the barium salt of *N*-acetyl L-thyroxine in net yields of 15–20%, calculated on the recovered starting material.

L-Thyroxine from *N*-acetyl L-thyroxine

N-Acetyl L-thyroxine (1.64 g) was boiled under reflux for 1 hr with 25 ml acetic acid and 15 ml conc HCl. The solution was then concentrated to dryness, and the residue was dissolved in boiling 0.1N Na_2CO_3 from which the sodium salt of thyroxine separated. After recrystallization, the thyroxine weighed 1.25 g and had *m.p.* 232° (decomp).

$[\alpha]_D^{21} - 5.40^\circ$ in 3.335% solution in two parts ethanol and one part λ -NaOH. Since this rotation was considerably higher than that found for L-thyroxine by Harington & Pitt-Rivers (1945), it was thought that the L-thyroxine obtained above might be contaminated by a small amount of N-acetyl L-thyroxine. A second hydrolysis with the same acid mixture was done for 2 hr. The L-thyroxine obtained had $[\alpha]_D^{21} - 5.42^\circ$ in 3.28% solution in the same solvent. The specific rotation of the L-thyroxine was then determined in 3.25% solution in a mixture of 24 g 0.5N NaOH and 56 g ethanol, and was found to be $[\alpha]_D^{17.6} - 4.62^\circ$. This is in good agreement with the value obtained by Foster, Palmer & Leland (1936) for L-thyroxine from the thyroid gland; these authors found $[\alpha]_D - 4.4^\circ$ in the same solvent.

Attempted identification of the side chain of diiodotyrosine which is eliminated during thyroxine synthesis

N-Acetyl L-diiodotyrosine (96 g) was dissolved in 1.12 l dilute baryta solution containing 1.78 equiv Ba(OH)₂/mol acetyldiiodotyrosine. The initial pH was 7.6 as measured by cresol red (British Drug Houses Ltd capillator). After 3 weeks at 35° the barium salt of acetylthyroxine, which had separated, was collected on the filter, and after decomposition with acid 6.23 g acetylthyroxine were obtained. The mother liquor was just acidified with dilute H₂SO₄. Much unchanged acetyldiiodotyrosine was precipitated together with BaSO₄, from which it was separated by solution in dilute alkali and reprecipitation with acid. The recovered acetyldiiodotyrosine weighed 63.0 g, whence the net yield of acetylthyroxine was nearly 22%. The filtrate was extracted with ether several times to remove some acetyldiiodotyrosine which has a significant water solubility. It was concentrated somewhat and made 2N with respect to H₂SO₄ and boiled under reflux for 1 hr to hydrolyze any acetyl amino acid present. The solution was then freed from H₂SO₄ with Ba(OH)₂ and concentrated to about half its volume. Addition of Ag₂SO₄ in excess precipitated the inorganic iodide liberated by decomposition of acetyldiiodotyrosine during the incubation. Sulphate ions were again removed with Ba(OH)₂, and the filtrate was concentrated to 200 ml. At this point, the HNO₃ reaction for *o*-diiodophenolic groups was positive, as was the ninhydrin reaction. It was therefore suspected that diiodotyrosine was present, so the solution was treated with excess lead acetate and allowed to stand for 12 hr. The bulky precipitate was then separated on the filter and the filtrate freed from Pb with H₂S. After concentration to a small volume, crystals separated which had m.p. 215–216° (decomp). These crystals gave positive ninhydrin, Millon and HNO₃ reactions. The mixed melting point with authentic L-monoiodotyrosine was not depressed. The presence of this amino acid is attributed to incomplete iodination of the tyrosine from which the N-acetyl L-diiodotyrosine was prepared. Since monoiodotyrosine is a fairly soluble amino acid, it was thought that its removal would best be effected by its hydrogenation to tyrosine, which was accordingly done using palladized strontium carbonate as catalyst. After the reduction the solution was acidified with H₂SO₄ and freed from iodide with Ag₂SO₄, it was then freed from sulphate ions, and concentrated to about 15 ml. After several days in the ice chest 0.52 g of tyrosine had separated. This was removed, and the mother liquor (16 ml) was

examined by Dr R. L. M. Synge by paper chromatography. The predominant amino acid was alanine, a little tyrosine was also present. Attempts to crystallize the alanine by concentrating the solution to a low volume and distilling in ethanol were unsuccessful.

In a second experiment, 26.85 g N-acetyl L-diiodotyrosine were incubated at 35° in 415 ml baryta solution at pH 7.6 for 4 weeks. At the end of this time 1.69 g acetylthyroxine were obtained, and after removal of unchanged starting material (15.6 g) the filtrate was treated as in the first experiment. No monoiodotyrosine was detected. When the volume of the mother liquor had been concentrated to 25 ml, this was again examined by Dr Synge who found as before that alanine was the predominant amino acid present. Attempts to isolate alanine as the azobenzenesulphonic acid salt also failed.

Experiments on the incubation products of N-acetyl-DL-diiodotyrosyl L-glutamic acid

N-Acetyl DL-diiodotyrosyl L-glutamic acid 2-Methy-14(3' 5' diiodo-4'-acetoxybenzyl) oxazolone (Gell, Harington & Pitt-Rivers, 1946) (15.5 g) was dissolved in 120 ml acetone and mixed with a solution of glutamic acid (7.35 g) in 100 ml λ -NaOH. After 12 hr at room temperature 31 ml 2N NaOH were added to the solution to hydrolyze the O-acetyl group. After 1 hr acetone was removed under diminished pressure, the solution was diluted with water and acidified with 3N HCl, the product, which crystallized in small prisms, was recrystallized by dissolving in warm methanol and diluting with water to turbidity, the yield was 14.2 g (76% of the theoretical). The compound had m.p. 212–214° (decomp). (Found C, 30.8, H, 3.47, I, 40.9. C₁₈H₁₈O₇N₂I₂ · H₂O requires C, 30.9, H, 3.22, I, 40.8%). On attempting to dehydrate this compound by drying *in vacuo* at 100°, about 5% of its weight was lost, and there was some decomposition.

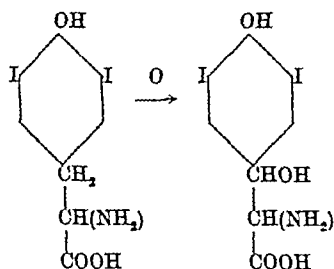
The isolation of N-acetyl DL-thyroxyl L-glutamic acid from the incubation products of the above peptide. Some preliminary incubations were done on N-acetyldiiodotyrosylglutamic acid in dilute NaOH solution at pH 7.2 and hydrolysis of the reaction mixture yielded some thyroxine, however, no insoluble sodium salt of the thyroxine derivative separated during the incubations. N-acetyl DL-diiodotyrosyl L-glutamic acid (11.8 g) was dissolved in 155 ml 0.341N Ba(OH)₂, the pH was 7.4 as measured with indicators. After a few hours at 37° a partly crystalline barium salt had started to separate and this greatly increased in the course of time. After 4 weeks, the barium salt was collected by filtration, washed with water and decomposed with dilute HCl. The product (2.3 g) was crystallized by dissolving in cold methanol and diluting with water to turbidity, and separated in minute prisms which had m.p. 190–200° (decomp). (Found C, 27.8, H, 2.77, I, 52.5. N-acetylthyroxylglutamic acid, C₂₂H₂₀O₈N₂I₄ · H₂O requires C, 27.3, H, 2.3, I, 52.6%). Attempts to dehydrate this compound at 100° led to some decomposition and loss of iodine. This thyroxine derivative does not form an insoluble sodium salt. Of N-acetyl DL-diiodotyrosyl L-glutamic acid 3.44 g were recovered from the mother liquor, whence the net yield of the thyroxine derivative was 36%.

Acid hydrolysis of N-acetyl DL-thyroxylglutamic acid. The peptide (1.93 g) was boiled under reflux for 1.5 hr with acetic acid (25 ml) and conc HCl (15 ml). The solution was concentrated to dryness and the residue was extracted with

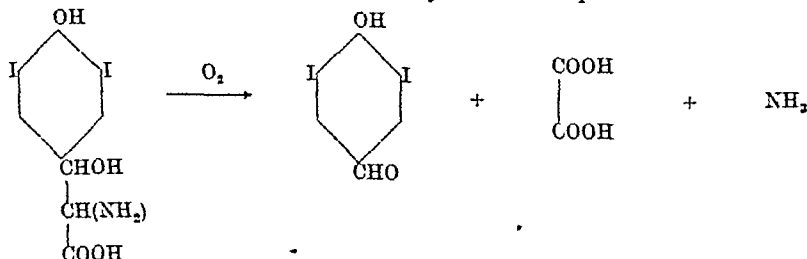
dilute HCl. The insoluble thyroxine was collected and purified in the usual manner. After crystallization the yield of thyroxine was 0.77 g and it had *m p* 235° (decomp). The filtrate was concentrated to a low volume and brought to pH 3.2 with NH_3 . After a short time, triangular prisms began to separate. After 24 hr in the ice chest these were collected, and the mother liquor was concentrated when more of the same crystals separated. The total yield of this compound was 0.163 g. After crystallization from water the compound had *m p* 204° (decomp). The mixed melting point with authentic L-glutamic acid was not depressed. (Found: C, 40.9, H, 6.0, N, 9.7. Calc. for $\text{C}_8\text{H}_9\text{O}_4\text{N}$: C, 40.85, H, 6.1, N, 9.5%.)

DISCUSSION

The isolation of the side chain of diiodotyrosine as a 2 carbon residue (oxalic acid), after the formation of 3,5-diiodo-4-hydroxybenzaldehyde during oxidation at high pH values, suggests immediately the conclusion that the first step in the degradation of the amino acid side chain under these conditions entails oxidation on the β carbon atom, with the formation of 3,5-diiodo-4-hydroxyphenylserine. By analogy with the finding of Bettzieche (1925), that phenylserine is broken down in alkaline solution to give benzaldehyde and glycine, the above hydroxy acid would, under the same conditions, give 3,5-diiodo-4-hydroxybenzaldehyde and glycine.



Now it is a little difficult to believe that the glycine molecule thus produced would, under the mild oxidative conditions prevailing during aerobic incubation at 38°, be oxidized quantitatively to oxalic acid (cf. Dakin, 1905-6, on the formation of glyoxylic acid and oxalic acid by the action of hydrogen peroxide on glycine). It is, therefore, suggested that the 3,5-diiodo-4-hydroxyphenylserine undergoes further oxidation on the α or β carbon atom, and subsequent fission to the aldehyde, oxalic acid and ammonia.



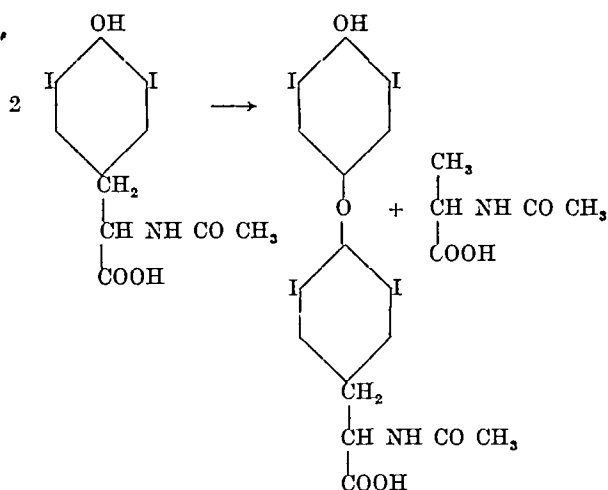
With regard to the formation of 3,5-diiodo-4-hydroxybenzaldehyde during the preparation of artificial thyroproteins, this may be simply a function of the pH of the reaction. The absence of this aldehyde from the thyroid is exceedingly probable, but has not yet been demonstrated conclusively, since the sample of B.P. thyroid used for an attempted isolation had been defatted, and any aldehyde present would be removed by any fat solvent. The experiment with thyroid powder was done in order to find out whether the aldehyde obtained from the iodinated casein might be an artifact of alkaline hydrolysis from diiodotyrosine present in the protein, and the negative result with thyroid powder showed that this was not so. For other reasons, however, it is unlikely that the aldehyde is formed in the thyroid gland *in vivo* first, because the pH of the tissues is below that at which aldehyde formation occurs during the incubation of diiodotyrosine, and secondly, because Harington & Randall (1929) were able to account for all the acid-insoluble iodine in the thyroid as being thyroxine iodine.

The formation of 2,4,6-triodophenol from diiodotyrosine after hot peroxide oxidation calls for some comment. It has been found, during a repetition of Dakin's (1909) experiments on the formation of quinols from hydroxybenzaldehydes, that 3,5-diiodo-4-hydroxybenzaldehyde, while it gives no quinol with hydrogen peroxide, can, under vigorous conditions of oxidation, lose the formyl group with the production of 2,4,6-triodophenol besides much free iodine and iodoform. No triiodophenol is formed from diiodotyrosine under mild oxidative conditions, which indicates that the triiodophenol obtained from diiodotyrosine during hot peroxide oxidation is a secondary breakdown product formed from the aldehyde, the formation of this compound is, therefore, not considered to have any importance in elucidating the mechanism of thyroxine synthesis.

The original suggestion by Harington & Barger (1927) that thyroxine was formed by the coupling of two molecules of diiodotyrosine was amplified first by Johnson & Tewkesbury (1942). These authors postulated that during incubation of diiodotyrosine under von Mutzenbecher's (1939) conditions hypiodite is formed. This oxidizes one molecule of diiodotyrosine to a quinonoid intermediate possessing a

free radical, which unites with a further molecule of diiodotyrosine with univalent oxygen, the side chain being split off as pyruvic acid and ammonia. Later Harrington (1944) developed a theoretical mechanism for thyroxine synthesis based on electronic considerations, and leading to the same type of intermediate as that suggested by Johnson & Tewkesbury (1942).

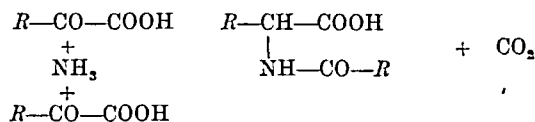
The chromatographic identification of small amounts of alanine among the reaction products obtained by acid hydrolysis of the mother liquors, after acetylthyroxine has been formed from acetyldiiodotyrosine, may lead to a different mechanism. If we suppose that this alanine is derived from the side chain which has been eliminated from one molecule of acetyldiiodotyrosine during the coupling, then the overall reaction may be graphically represented as a dismutation



No oxidative process is required for this representation, although it is known (Harrington & Pitt Rivers, 1945) that thyroxine is not formed from diiodotyrosine under anaerobic conditions, and iodine is, in fact, liberated from acetyldiiodotyrosine during the reaction. This can be demonstrated by the presence of inorganic iodide in the reaction mixture, even in experiments where the net yields of acetylthyroxine are high and the recovered acetyldiiodotyrosine is free from tarry products (i.e. incubation in borate buffer).

The presence of alanine and the absence of serine (and glycine) during thyroxine synthesis recall the metabolism of tryptophan by *Escherichia coli*, elucidated by Dawes, Dawson & Happold (1947). These authors have identified the tryptophan side chain chromatographically as alanine when indole is formed by *Esch. coli* in the presence of the retarding agent mepacrine, and no serine has been found. It is interesting to speculate whether the two reactions, resulting in thyroxine on the one hand, and indole on the other, may not follow the same metabolic

pathway, although the causative factor is different in the two cases, the former being chemical, the latter enzymic. Acetylalanine might, on the other hand, be obtained as a by-product of an entirely different reaction. If we assume that Johnson & Tewkesbury's (1942) mechanism of thyroxine synthesis is correct, the side chain would be eliminated from acetyldiiodotyrosine as pyruvic acid and acetamide. Now Knoop's theory (Knoop, 1910, Knoop & Kertess, 1911) to explain the formation of amino acids from keto acids *in vivo* postulated the intermediate formation of an acylated amino-acid



This theory was based on Knoop's own findings from *in vivo* experiments and was supported by the work of Erlenmeyer & Kunlin (1899, 1902) and de Jong (1900), who studied the *in vitro* reaction with many keto acids, in particular, when pyruvic acid and ammonium carbonate reacted together in aqueous solution, acetylalanine was formed in good yield. The theory was much later re-established by the work of du Vigneaud & Irish (1938) in experiments on the transformation in dogs of one amino acid isomer into its enantiomorph through the intermediate keto acid.

If this be the source of acetylalanine in the reaction at present being studied, it must be assumed that the acetamide liberated during deamination of the side chain is hydrolyzed to acetic acid and ammonia, since Herbst (1939) has shown that the product of condensation of pyruvic acid and acetamide itself is of an entirely different nature. One experimental result, however, can decide upon the genesis of the alanine which has been detected. Acetylalanine obtained *in vitro* from pyruvic acid and ammonia would be optically inactive, whereas alanine split off as acetylalanine from *N*-acetyl diiodotyrosine would be optically active, as is the *N*-acetylthyroxine formed. Efforts are now being made to isolate either acetylalanine or alanine itself in sufficient quantity to determine this point. Until this is done, the mechanism of the formation of alanine during thyroxine synthesis cannot profitably be discussed.

SUMMARY

1. 3,5-Diiodo-4-hydroxybenzaldehyde and oxalic acid have been isolated in equimolecular amounts after aerobic incubation of diiodotyrosine at pH 14. These findings are discussed.

2. The above aldehyde and thyroxine are formed together during aerobic incubation of diiodotyrosine at pH 10.

3 2 4 6 Triiodophenol, the aldehyde and thyroxine are all formed during the hot peroxide oxidation of diiodotyrosine

4 3 5 Diiodo 4 hydroxybenzaldehyde is formed during the iodination of proteins to give products with thyroid like activity, the aldehyde itself has no biological activity as measured by the tadpole metamorphosis test

5 *N* Acetyl L thyroxine is formed in substantial yield during aerobic incubation of *N* acetyl-L diiodotyrosine. Acid hydrolysis of the thyroxine derivative yields L thyroxine

6 *N* Acetyl DL diiodotyrosylglutamic acid has been prepared. Aerobic incubation of this peptide leads to the formation of good yields of *N* acetyl

DL thyroxylglutamic acid which, on acid hydrolysis, yields thyroxine and glutamic acid

7 Alanine has been chromatographically identified among the reaction products obtained during thyroxine synthesis from diiodotyrosine. This finding is briefly discussed.

I wish to express my indebtedness to Dr C R Harrington for his interest in this work. I have to thank Dr R L M Syngé for doing the chromatographic identifications of alanine, Dr W J Elford for his help in doing the potentiometric titration of *N* acetyl L-diiodotyrosine, and Dr A S Parkes for testing the biological activity of 3 5 diiodo 4-hydroxybenzaldehyde. I have also to thank Imperial Chemical Industries (Explosives) Ltd for gifts of iodinated proteins.

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The Steroids of Pregnant Mares' Urine

1 A METHOD FOR THE EXTRACTION OF STEROID SULPHATES AND THE ISOLATION OF *allo*PREGN-16 EN 3(β) OL 20 ONE SULPHATE*

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(Received 23 October 1947)

In a previous paper (Schachter & Marrian, 1938) dealing with the isolation of oestrone sulphate from the urine of pregnant mares, the isolation from the same source of what appeared to be the sulphate of a non phenolic steroid was briefly noted. Fuller details were not reported at that time since concordant analyses upon the new sulphate and the parent steroid could not be obtained, and since there

* For preliminary accounts of this work see *Biochem J* 1945, 39, xiv, 1946, 40, iv, 1948, 42 i

was some evidence to suggest that the material isolated might be a mixture of closely related sulphates varying in composition from batch to batch. More recently, it has been possible to commence a reinvestigation of the conjugated steroids in pregnant mares' urine, and in the present paper the methods used for the extraction and concentration of a steroid sulphate fraction and the isolation and identification of one pure component of this fraction are described.

RESULTS

The method used for the extraction and concentration of the steroid sulphate fraction was based on that of Schachter & Marrian (1938). It consisted of the following stages: (i) Extraction of the urine with *n* butanol; (ii) Removal of much acidic matter from the material extracted with butanol, giving the 'crude conjugate fraction'; (iii) Treatment of an aqueous solution of the crude conjugate fraction with an equal volume of saturated potassium acetate, giving 'potassium acetate soluble' and 'potassium acetate insoluble' fractions. This stage served the double purpose of ensuring that K^+ was the only cation present in the insoluble fraction and of partially removing in the soluble fraction certain simpler organic sulphates such as that of *p* cresol; (iv) Separation of the 'potassium acetate insoluble' fraction into fractions readily and sparingly soluble in water, so called 'water soluble' and 'water insoluble' fractions; (v) Repeated extraction of the 'water-insoluble' fraction with boiling acetone containing water (2% (v/v), '98% acetone'). On concentration and cooling to 0° these extracts yielded white solid material, '98% acetone solids, fraction C', which in some cases was crystalline. (In one batch of urine solid material (fraction A) separated from the 98% acetone extracts before concentration on cooling to 0°. In another batch, although no solid was obtained on cooling the unconcentrated extracts, crystalline material (fraction B) separated immediately when these extracts were heated in order to concentrate them. Fractions A and B will be dealt with in subsequent papers.)

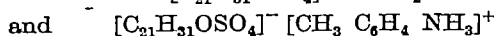
The weights of certain fractions at the different stages of concentration for a number of batches of urine are summarized in Table 1.

Table 1 *Weights of conjugate fractions in standard extraction method*

(All batches of urine from mares in 9th-11th months of pregnancy. Results expressed as mg/l. urine.)

	No. of results	Mean	S.E. of mean	Range
Crude conjugate fraction	11	440	50	210-810
Potassium acetate soluble fraction	8	40	8	20-80
Potassium acetate insoluble fraction	10	280	30	140-450
Water soluble fraction	2	175	—	160-190
Water insoluble fraction	10	120	20	50-210

Fraction C from certain batches of urine was found to consist largely of a single substance (*potassium Z sulphate*), which was obtained pure either directly by crystallization from water and from ethanol or via its *p* toluidine salt (cf. Barton & Young, 1943). Analyses indicated the formulae



for the potassium and *p* toluidine salts, respectively.

On acid hydrolysis of the potassium or *p*-toluidine salt an ether soluble substance (*Z*), the analysis of which agreed with the formula $C_{21}H_{32}O_2$, was obtained. *Z* yielded a monoacetate on treatment with acetic anhydride and pyridine, and gave a precipitate with digitonin in 90% (v/v) ethanol. It thus appeared to be a 3(β) hydroxysteroid. No colour was given with tetranitromethane.

In the Zimmermann test *Z* gave a purple colour, although the development of the colour was slower than with neutral 17-ketosteroids, indicating the presence of a ketone group at some position other than C_{17} . *Z* did not, however, form a semicarbazone. The failure of *Z* to form a semicarbazone and the lack of colour with tetranitromethane were not inconsistent with the presence of an $\alpha\beta$ unsaturated ketone group (cf. Ruzicka, Huyser, Pfeiffer & Seidel (1929) on tetranitromethane reactions, and Allen & Blatt (1943) on semicarbazide reactions), and subsequently *Z* acetate was found to show the 240 and 320 $m\mu$ absorption bands characteristic of $\alpha\beta$ unsaturated ketones.

The above evidence suggested that *Z* might be a pregnen- or *allopregnen*-3(β)-ol one having the double bond conjugated with the ketone group, its melting point and that of its acetate indicated that it might be *allopregn* 16 en 3(β)-ol 20 one, which was obtained by Marker & Turner (1940) from dihydropseudotigogenin diacetate.

Samples of this *allopregnenolone* acetate and of the 16 17-epoxy compound obtained from it by treatment with perbenzoic acid (Plattner, Ruzicka, Heusser & Angliker, 1947) were kindly supplied by Prof. Plattner of Zurich, and a comparison of these samples with *Z* acetate and its epoxy compound, respectively, showed that *Z* was indeed *allopregn* 16 en 3(β)-ol 20 one. This appears to be the first reported isolation of a Δ^{16-20} ketosteroid from urine.

The batches of urine from which *Z* sulphate was readily isolated were collected 3 weeks or less before foaling, the yield of crude potassium *Z* sulphate being c. 25 mg/l. Other batches collected 4-16 weeks before foaling gave smaller quantities of crude material, from which it was more difficult to obtain *Z* sulphate. This suggests that the amount of *Z* sulphate excreted, and the ratio of *Z* sulphate to related sulphates, both increase in the last few weeks before foaling.

EXPERIMENTAL

Melting points These are corrected Speed of heating at melting point, 3–4°/min

Micro analyses We are indebted to Mrs G F Marrian for all micro analyses, except those of compound Z which are by Drs Weiler and Strauss (Oxford)

Specific rotations These are for the sodium D line, a 0.5 dm micro tube was used $[\alpha]_D$ is given as

$$X^\circ \pm Y^\circ = \frac{100}{lc} (x^\circ \pm y^\circ),$$

where x° is the difference between the mean of twenty readings with the solution and the mean of twenty readings with a solvent blank, y° is the standard error of this difference Allowance is thus made for the variation of the polarimeter readings, but not for errors in the weighing of solute or solvent, which are much smaller

Extraction of urine and preparation of fraction C Many modifications of the extraction procedure were tried (see Klvne, 1946) The following are details for a batch of urine from which Z sulphate was obtained

Urine (22.2 l., collected in the last 3 weeks before foaling) was extracted with *n* butanol (4 × 6 l.) Emulsions were broken by allowing to stand with gentle shaking, centrifuging or adding ethanol (1–5% of vol of urine) The butanol extracts were washed with *n* aqueous KOH (4 × 6 l.) and with water (4 × 1.5 l.) Emulsions in the water washing stage were broken rapidly by the addition of saturated aqueous potassium acetate (10% of vol of aqueous layer)

The washed butanol extracts were evaporated to dryness *in vacuo* at $\approx 100^\circ$ and the residue dissolved in water (500 ml.) The aqueous solution so obtained was extracted with ether (4 × 200 ml.) and cooled to 0° , its pH was adjusted to c 2 with HCl and it was again extracted with ether (2 × 300 ml., 4 × 200 ml.) It was then made alkaline to litmus with KOH, warmed to remove ether, cooled, and extracted with butanol (4 × 200 ml.) The butanol extracts were washed with water (3 × 100 ml.) and evaporated to dryness giving the 'crude conjugate fraction' as a brown friable powder (12.3 g.)

This material was dissolved in boiling water (90 ml.), saturated aqueous potassium acetate (90 ml.) was added, the mixture was cooled to 15–20° and the pasty precipitate centrifuged The precipitate was washed twice by centrifuging with its own volume of half saturated aqueous potassium acetate and dissolved in moist butanol (250 ml.) This solution was washed with water (3 × 50 ml.) and evaporated to dryness giving the 'potassium acetate insoluble fraction' as a yellow powder (8.8 g.)

The supernatant and washings from the potassium acetate precipitation were extracted with butanol (4 × 100 ml.), the extracts were washed with water (3 × 50 ml.) and evaporated to dryness giving the 'potassium acetate soluble fraction' as a nearly white powder (2.0 g.)

The 'potassium acetate insoluble fraction' was dissolved in the minimum volume of boiling water (140 ml.) and cooled to 0° The sticky precipitate which formed was centrifuged, washed once by centrifuging with its own volume of ice cold water and dissolved in moist butanol (200 ml.) The butanol solution on evaporation to dryness gave the water insoluble fraction as a yellow powder (4.7 g.)

The supernatant and washings from the above separation were extracted with butanol (3 × 100 ml.), the extracts were washed with water (3 × 20 ml.) and evaporated to dryness giving the 'water soluble fraction' as a pale brown powder (3.9 g.)

The 'water insoluble fraction' was then extracted repeatedly with boiling 98% acetone (2 × 200, 6 × 100 ml.) Each extract was filtered hot and the undissolved solid used for the next extraction The extracts, which on cooling gave no precipitate, were concentrated on the water bath under a gentle air blast until solid began to separate, on cooling again, a nearly white, partly crystalline precipitate separated (98% acetone solids, fraction C, 620 mg.) The melting points of the fractions ranged from 218–219° (decomp) (first fraction) to 235–240° (decomp) (last three fractions)

Isolation of Z sulphate as the *p* toluidine salt The 98% acetone solids, fraction C, from the above separation (301 mg.) were dissolved in boiling water (50 ml.) and a solution of *p* toluidine hydrochloride (185 mg., 2 equiv.) in water (5 ml.) was added The copious precipitate which formed was just dissolved by adding more water (35 ml.) at 100° , and the solution was cooled to 0° giving *p* toluidine Z sulphate (293 mg., m p 190–192°) After two recrystallizations from boiling water this salt formed very fine white needles, m p 195–197°, after softening at 186° , $[\alpha]_D^{19} + 31.7^\circ \pm 0.6^\circ$ in chloroform (c, 3) Approximate solubilities (in mg/ml) were as follows in water, at b p, 3.5, at 15–20°, 0.5, in chloroform, at 15–20°, >30 The salt showed no loss in weight on drying at 80° *in vacuo* (Found C, 67.2, 67.2, H, 8.1, 8.1, N, 2.8, 2.9, S, 6.4, 6.4 $[C_{21}H_{31}OSO_4]^- [CH_3C_6H_4NH_3]^+$ requires C, 66.8, H, 8.2, N, 2.8, S, 6.4%)

Potassium Z sulphate This was obtained from the 98% acetone solids, fraction C, from two batches of urine by repeated crystallization from water and then from ethanol It was best obtained pure from the *p* toluidine salt as follows

p Toluidine Z sulphate (100 mg.) in warm water (100 ml.) was treated with KOH (1.0 g.) in water (5 ml.), and *p* toluidine was extracted with ether (4 × 25 ml.) The aqueous solution was heated to drive off dissolved ether, cooled, and extracted with butanol (2 × 50, 2 × 25 ml.) The extracts on evaporation gave crude potassium Z sulphate, 86 mg. (90% of theory), m p 222–224° (decomp), which was then crystallized from water and from ethanol giving rosettes of needles, m p 244–245° (decomp) after browning at 234° and sintering at 241°

Analyses of the potassium salt, when compared with those of the *p* toluidine salt and of the free steroid, showed that it was a *monohydrate*, which did not lose its water of crystallization on drying to constant weight at 80° *in vacuo* over P_2O_5 (Found C, 55.8, 55.8, H, 7.5, 7.5, K, 8.8, 8.5 $C_{21}H_{31}OSO_4K \cdot H_2O$ requires C, 55.7, H, 7.4, K, 8.6 $C_{21}H_{31}OSO_4K$ requires C, 58.0, H, 7.2, K, 9.0%)

Approximate solubilities (in mg/ml) were as follows in water at b p, 10–11, at 0° , 3, in ethanol at b p, 9, at 0° , 2.5 in butanol at b p, 10, at 0° , 7, in chloroform at b p or at 0° , <0.1

The potassium salt was dimorphous, clusters of prisms, m p 216–217° (decomp) sometimes appearing when the compound was crystallized from water (The quantity obtained was insufficient for analysis) This prismatic form on treatment with *p* toluidine hydrochloride gave the *p* toluidine salt described above

Table 2 Comparison of *Z* acetate and allopregn-16 en 3(β) ol-20 one acetate

	<i>Z</i> acetate	alloPregnenolone acetate
M p	165-166.5°	165-167°*
Mixed m p		163-165.5°
$[\alpha]_D^{25}$ in chloroform (c, 1.4)	+33.9° ± 0.6°	+36.3° ± 0.7°*
Ultraviolet absorption†		
λ_{max}	240, 320 m μ	240, 320 m μ
log ϵ_{max}	3.95, 1.78	3.93, 1.92*
16.17 Epoxy compound		
M p	185-187°	183-185°*
Mixed m p		183-185°

* These values were determined on Prof. Plattner's samples in Edinburgh and Liverpool. Plattner *et al.* (1947) give the following values: allopregnenolone acetate, m p 166-167°, $[\alpha]_D^{20}$ +42.2° (c, 1.42, chloroform), log ϵ_{max} = 4.2, 2.1 (solvent not stated). Epoxy compound, m p 180-187°.

† In ethanol containing a little ether. We are indebted to Prof. R. A. Morton, Dr. A. L. Stubbs and Mr. T. W. Goodwin (University of Liverpool) for these measurements.

Compound Z. *p*-Toluidine *Z* sulphate (154 mg) was dissolved in hot water (100 ml.), 2N HCl (100 ml.) was added, and the mixture was heated in the boiling water bath. The initially clear solution became cloudy after 10 min. heating, and a precipitate formed, which gradually increased in bulk. After 2 hr. heating the mixture was cooled and extracted with ether (1 × 100 ml. and 3 × 50 ml.). The ether extracts were washed with N HCl (2 × 30 ml.), 0.5N NaHCO₃ (30 ml.) and water (3 × 30 ml.). The extracts on evaporation to dryness yielded crude compound *Z*, 88 mg (91% of theory), m p 197-202° after sintering at 184°. After two recrystallizations from aqueous ethanol, *Z* formed glistening leaflets, m p 205-207°, $[\alpha]_D^{17}$ +50.2° ± 4.2° in ethanol (c, 1.4) (Found C, 79.5, 79.8, H, 10.3, 10.1. Mol. wt. (Rast) 307, 325. Calc. for C₂₃H₃₂O₂, C, 79.7, H, 10.2%. Mol. wt. 316).

***Z* acetate.** Compound *Z* (7.8 mg) was heated at 100° for 2 hr. with acetic anhydride (1 ml.) and pyridine (1 ml.). Much water was added, and the white flocculent precipitate obtained was filtered, washed and dried. This material was recrystallized twice from aqueous ethanol (approx. 1 l. by vol.) giving pure *Z* acetate in elongated hexagonal leaflets, m p 165-166.5° (Found C, 76.7, 76.4, H, 9.5, 9.8. Calc. for C₂₃H₃₄O₃, C, 77.1, H, 9.6%).

The epoxy compound of *Z* acetate was prepared by treatment with perbenzoic acid as described by Plattner *et al.*

(1947). The properties of *Z* acetate and allopregn-16-en-3(β) ol-20-one acetate are compared in Table 2.

SUMMARY

1. A general method for the extraction of steroid and other conjugates from mares' urine is described.

2. A new organic sulphate has been isolated from pregnant mares' urine as its *p*-toluidine salt, which on acid hydrolysis yielded allopregn-16-en-3(β) ol-20-one.

We are indebted to the Agricultural Research Council for a grant which defrayed the greater part of the expenses of this work, also to the Moray Fund and the Lewis Cameron Fund of the University of Edinburgh for other grants. We are grateful to N. V. Organon, Oss, Holland, for the gift of a large volume of mares' urine and facilities for working this up, also to British Drug Houses Ltd., the Ovaltine Research Laboratories and the Animal Diseases Research Institute, Moredun, Edinburgh, for gifts of urine. Without their willing co-operation this research could not have been carried out. We are also indebted to Goodlass Wall and Lead Industries Ltd. for the loan of large scale apparatus, to Mrs. G. F. Marrian for micro analyses and to Mr. A. Purdie for much valuable help in stable and laboratory.

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The Effect of Thyroxine on the Metabolism of Lactating Cows

1 GENERAL RESULTS AND NITROGEN METABOLISM

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That thyroxine causes an increase in milk secretion and in the yield of milk fat has been shown by Graham (1934 *a, b*), Jack & Bechdel (1935), Folley & White (1936), Herman, Graham & Turner (1938) and Smith & Dastur (1940). These observations did not at first arouse general interest because of the prohibitive cost of thyroxine. Later, however, Ludwig & von Mutzenbecher (1939) showed that thyroid-active material could be produced from casein by iodination, and this was confirmed by Harington & Pitt Rivers (1939), who demonstrated that in the iodination thyroxine was formed from the tyrosine of the casein. A few years later the conditions of iodination were further defined by Turner & Reineke (1944) in such a way as to ensure reasonable yields of thyroxine.

Since these observations were made, the use of iodinated casein for increasing the milk yield of cows in commercial herds has been investigated, notably by Blaxter (1945, 1946). Blaxter's work leaves little doubt that, when cows are given iodinated protein in their feed under ordinary conditions of farm management, the extra milk and milk fat produced result not from an increase in the intake of food, but from raised catabolism. The animals lose condition, probably because the extra milk is formed, in part at least, from the proteins and fat of the animals' own tissues during a period when the metabolic rate of the animals is increased. As increased catabolism might have marked adverse effects on lactating cows, the work described in the present paper was designed to determine to what extent the stimulation of milk production by thyroxine is associated with loss of body tissue and with negative nitrogen balance, and whether increase of food intake can compensate for any raised catabolism which may occur. For all the experiments thyroxine was used rather than iodinated protein, because of the risk that the activity of iodinated casein given orally would vary greatly from sample to sample and be difficult to assess. This uncertainty does not exist if a known dose of thyroxine is given subcutaneously.

EXPERIMENTAL

General plan of the experiments and care of the animals
Three experiments were carried out, each involving three Ayrshire cows which, at the beginning of the experiments,

were 10–20 weeks from parturition. The animals were kept in special stalls in a metabolism house (Morris & Wright, 1933) in which the urine and faeces excreted by the cows can be collected separately with only slight mutual contamination. Before an experiment began, the cows were given a period of 2 or 3 weeks to accustom themselves to the metabolism house management and to the diets used in the experiment. This was followed by three periods, each lasting for at least 4 weeks. In the first and third periods no thyroxine was given, but in the second period two of the cows were treated with the hormone, the third cow receiving no thyroxine in any of the periods. In this way it was hoped to compare the findings obtained for the thyroxine treated cows during the second period of each experiment with the findings for the same cows before and after thyroxine treatment, and also with those for the control cow throughout the whole experiment. This was possible for the first two experiments, but during the third experiment the control cow became ill, too late to be replaced, and had to be discarded. All the animals were weighed at the beginning and end of each period. Every 2 days, samples of food, urine, faeces and milk were analyzed.

Diet In the first experiment the rations allowed per gallon of milk were purposely kept slightly below the lowest level which would ever be likely to be fed in practice. This resulted in negative N balances during the thyroxine period. In the second and third experiments the rationing was made progressively more liberal in order to determine how far the tendency to negative N balance could be overcome by increased food intake.

In the first experiment the maintenance ration consisted of 14 lb hay and 1 lb bruised oats/cow/day, and the production ration of 3 13 lb bruised oats and 0 91 lb bean meal (*Vicia faba*) per gallon of milk. The amount of production ration fed per day in any week was adjusted according to the yield of milk in the preceding week. The same production ration was fed in the second experiment but at a slightly higher level. In the third experiment the rations contained cubes consisting of wheat feed 42.5%, decorticated groundnut 27.5%, oats 20% and molasses 10%. For the first few weeks of the first control period of this third experiment, the maintenance ration was 14 lb hay/day and for production a mixture was made up consisting of equal parts of bruised oats, bean meal and cubes. 4 lb of the mixture being fed per gallon of milk. Later in this first control period, in order to ensure that the animals would be liberally fed just before and during the thyroxine treatment, the allowance of hay was raised to 16 lb and the concentrate mixture was changed to oats (3 parts), bean meal (3 parts) and cubes (4 parts), and fed at the rate of 5 lb per gallon of milk on the basis of the yield during the previous fortnight. This basis of rationing was continued for the remainder of the experiment.

In work of this type, where attempts are made to feed rations strictly according to milk yield, it is not easy to be certain that the intended relationship between milk production and protein intake will be accurately maintained, since quite unpredictable variations in milk yield tend to occur. The best way of ascertaining what the relationship actually was, is to calculate it at the end of the experiment from the figures obtained for the milk yield and for the actual amount of protein eaten. Data obtained in this way for the amount of protein fed per gallon of milk are shown in Table 1.

Table 1 *Protein equivalent ingested (lb/gallon of milk yielded)*

Exp no	Cow no	Period 1 (no thyroxine)	Period 2 (thyroxine)	Period 3 (no thyroxine)
1	1	0.51	0.50	0.77
	2 (control)*	0.44	0.41	0.44
	3	0.52	0.39	0.65
2	4	0.58	0.57	1.00
	5 (control)*	0.53	0.56	0.55
	6	0.50	0.52	0.95
3	7	0.62	0.68	0.87
	8	0.66	0.73	1.03

* The control cows (nos 2 and 5) received no thyroxine in period 2.

Administration of thyroxine A solution of 'thyroxine sodium B.P.' (British Drug Houses Ltd) containing 1 mg/ml was made up according to Folley & White (1936). Each day during the thyroxine periods 10 ml of this solution were injected subcutaneously into the flanks of the cows. Cows 1, 3, 4 and 6 were injected for 28 successive days, and cows 7 and 8 for 32 and 31 successive days respectively.

Methods of analysis Total N was estimated by the Kjeldahl method, the catalyst being K_2SO_4 , $CuSO_4$ and powdered selenium. For estimating N in milk, the samples were weighed and not pipetted. Milk fat was estimated by the Gerber process, and milk phosphatase by the method of Kay & Graham (1933). In the first two experiments the amount of urea plus NH_3 in the urine was determined

according to Peters & Van Slyke (1932), and in all three experiments the creatine and creatinine contents of urine were estimated by a modification of Jaffe's picric acid method (Hawk & Bergeim, 1927) using a Spekker absorptio meter.

RESULTS

Pulse rates and milk phosphatase It was essential for the purpose of this investigation to find out as early in the thyroxine period as possible whether the hormone was showing its usual physiological activity. The two criteria used for this purpose were the effect of thyroxine in enhancing the pulse rate and the rapid diminution which thyroxine produces in the concentration of phosphatase in the milk (Folley & White, 1936). Every day, except Sunday, throughout all three experiments pulse rates were determined by palpation of the plantar arteries of a forelimb, care being taken to ensure that the animals were not excited in any way. During the determination the animals were standing up and either eating hay or ruminating just after having eaten it. Four concurrent determinations were made. From the average results (Table 2) it can be seen that with all the treated animals there was a marked increase in pulse rate during the thyroxine period, whereas with the two controls during the same period no significant increase occurred. Table 2 also shows that the time, which elapsed from the first injection to the day when the pulse rate was maximal, varied from cow to cow. With cow 8 the pulse continued to increase during treatment. With all the other treated cows the pulse had passed its maximum before injection ceased.

Averages of many determinations of milk phosphatase are shown in Table 2. With the two controls there was a tendency for the phosphatase in the milk to increase as lactation advanced, whereas with the treated cows there was the expected substantial decrease during hormone treatment.

Table 2 *The effect of thyroxine on the pulse rate and on the phosphatase content of the milk*

Exp no	Cow no	Pulse rate/min				Phosphatase in milk*		
		Period 1 (no thyroxine)	Period 2 (thyroxine)		Period 3 (no thyroxine)	Period 1 (no thyroxine)	Period 2 (thyroxine)	Period 3 (no thyroxine)
			Days to maximum†					
1	1	46	65	17	49	68	31	84
	2 (control)	51	47	—	46	90	108	111
	3	59	68	6	52	22	7	43
2	4	65	81	19	66	47	8	39
	5 (control)	64	65	—	63	76	110	106
	6	55	60	13	55	39	8	53
3	7	57	90	29	69	136	40	164
	8	62	83	31	61	82	39	154

* Units of Kay & Graham (1933) multiplied by 100.

† Time elapsing from beginning of treatment to the day when the pulse was maximal.

Milk yield The milk-yield curves for all eight cows are shown in Fig 1, and the averages for each period in Table 3. It can readily be seen that with the two controls (nos 2 and 5) the milk yield tended to decrease gradually over the whole period, whereas with the remaining six cows the milk yield was enhanced from about 2 days after hormone injections began until a few days after they ceased. Owing to the difficulty of judging what the yield would have been had no hormone been given, it is not easy to determine quantitatively the effect of thyroxine on milk yield. From the graphs in Fig 1, however, it has been estimated that the average enhancement

of yield by thyroxine was about 30%, which is comparable to results reported by other workers mentioned in the introduction.

Milk composition With all the treated animals the yield of milk fat was greatly increased during the thyroxine period. Typical results for one control and one treated animal are shown in Fig 2. With all the treated animals the increase in the yield of fat persisted longer than that of milk as a whole after thyroxine treatment had ceased. The average values for the fat content of the milk and for the nitrogen content of the fat free milk from all eight cows, and for the non fatty solids for two of the cows, are

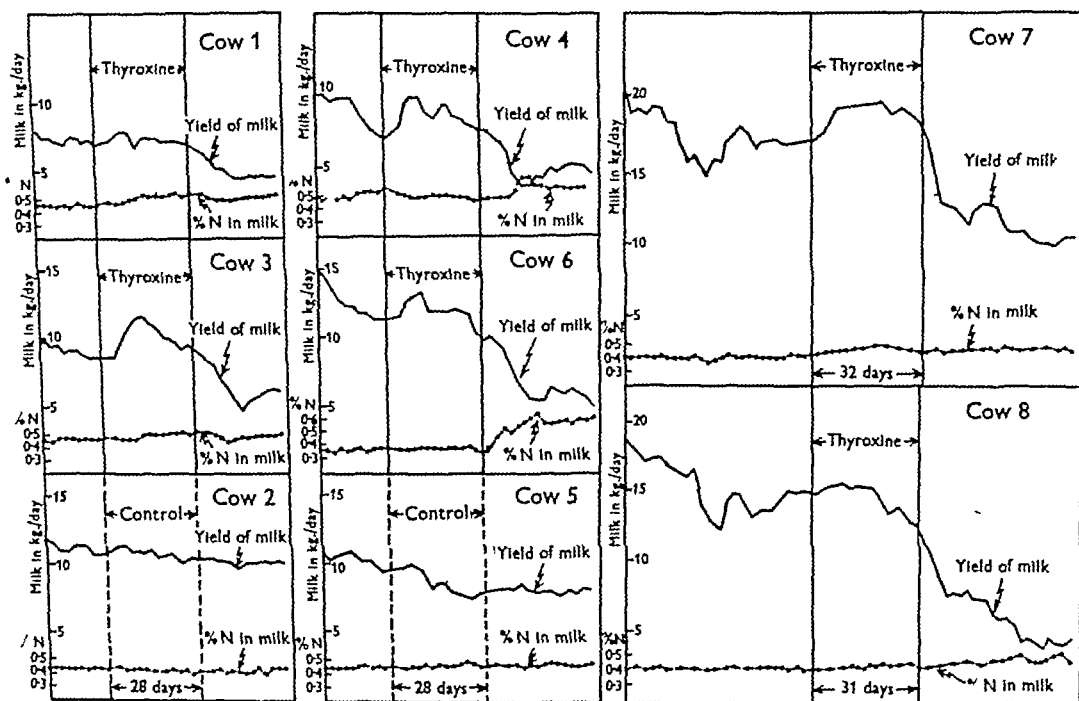


Fig 1 The effect of thyroxine on milk yield and on the percentage of nitrogen in the milk

Table 3 The average yield and composition of the milk produced in each period

(Thyroxine was given only during period 2 to all cows, with the exception of nos 2 and 3 which received no hormone at all)

Exp no	Cow no	Yield of milk (lb/day)			Fat content (%)			N content of the fat-free milk (%)			Solids content of the fat-free milk (%)		
		1	2	3	1	2	3	1	2	3	1	2	3
1	1	7.3	7.3	4.7	4.26	5.65	4.07	0.485	0.529	0.512	—	—	—
	2 (control)	11.2	10.8	10.0	3.63	3.32	3.15	0.451	0.427	0.431	—	—	—
	3	9.3	10.3	6.2	3.79	4.63	3.78	0.484	0.513	0.495	—	—	—
2	4	9.1	8.7	4.7	4.15	4.52	4.38	0.522	0.498	0.563	—	—	—
	5 (control)	10.3	8.5	8.0	2.98	3.10	2.95	0.453	0.477	0.485	—	—	—
	6	12.5	11.6	6.1	4.41	5.19	4.98	0.426	0.504	0.601	—	—	—
3	7	17.6	19.4	12.5	3.56	4.01	3.52	0.434	0.494	0.488	9.11	9.66	9.37
	8	15.2	14.6	6.7	3.70	4.08	4.41	0.441	0.465	0.522	8.77	9.06	9.00

shown in Table 3 Statistical analysis showed that the increases which occurred during hormone treatment were significant

Body weights All the animals were weighed at the beginning and end of each period Each weight recorded was the average of three weighings made

gained weight again in period 3, after thyroxine injections had ceased The result of this gain in weight in the final period was that the net loss of weight during the thyroxine and post thyroxine periods taken together (periods 2 and 3) was of the same order for nearly all the animals whether

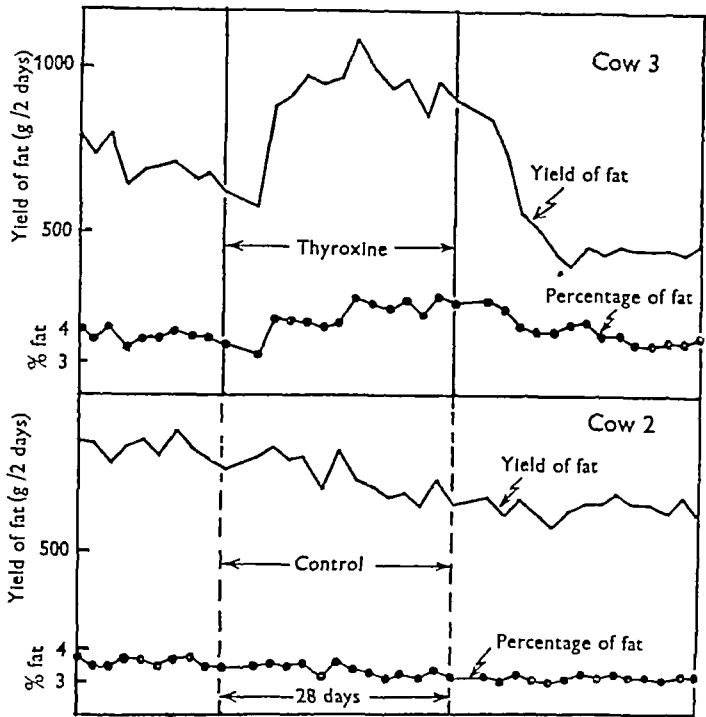


Fig 2 The effect of thyroxine on the yield and percentage of fat in the milk

Table 4 *Effect of thyroxine on body weight*

(Thyroxine was given during period 2 to all cows except the controls)

Exp no	Cow no	Weight at beginning of period 1 (lb)	Change in weight (lb) in each period expressed as % of the weight at the beginning of period 1			Change in weight (lb) in period 2 which was not made good in period 3 (b) + (c)
			Period 1 (a)	Period 2 (b)	Period 3 (c)	
1	1	857	-5.4	-5.8	+4.1	-1.7
	2 (control)	817	0.0	+2.1	-4.2	-2.1
	3	872	-0.4	-6.9	+6.3	-0.6
2	4	823	+0.2	-9.7	+8.7	-1.0
	5 (control)	1006	-0.9	+2.0	-1.3	+0.7
	6	945	-0.9	-13.3	+11.1	-2.2
3	7	1007	+0.2	-10.1	+3.2	-6.9
	8	1062	+7.5	-7.8	+6.8	-1.0

at different times on one day (Owen, Smith & Wright, 1943) The weights of the animals at the beginning of the first period of each experiment are shown in Table 4, the changes in weight which occurred during each period being expressed as a percentage of the initial weight It will be observed that, in contrast to the control cows, all the treated animals lost weight in the thyroxine period, but

treated or controls The only exception was cow 7 which did not show the same degree of recovery in period 3 as the others, although it, like cow 8, was fed the most liberal diet It should be noted, however, that cow 7 gave a greater increase in the yield of milk fat than cow 8 in the thyroxine period, and that this may have accounted in part for its lower recovery of weight

As is shown in the next paragraph, the nitrogen balances of cows 7 and 8, unlike those of the other treated cows, did not become negative when the cows were given thyroxine. It is, therefore, difficult to relate the loss in weight occurring during thyroxine treatment to any definite metabolic function. It is probable that the decreases of body weight of cows 1, 3, 4 and 6 were due to loss of both protein and fat, whilst the decreases of body weight shown by cows 7 and 8 were due mainly to loss of fat. Catabolism of fat need not parallel that of protein, while loss of protein from the body is known to be sometimes accompanied by retention of water, as, for example, in famine oedema. With ruminants the percentage of food in the gut is about 12% (Ritzman & Benedict, 1938), so that changes of 'fill' of the cows may have been partly responsible for changes of weight.

Nitrogen metabolism. All foods, including hay, and also milk, urine and faeces, were analyzed for total nitrogen every 2 days. Average results for each period are recorded in Table 5.

animals, cow 3 showed the greater stimulation of milk yield by thyroxine, and it also showed the greater negative nitrogen balance (-11.5 , as compared with -3.5 g N/day). After hormone treatment ceased, the nitrogen balances of both the experimental animals became markedly positive. In Exp 2, the results were very similar, in spite of the fact that the cows were allowed a slightly higher protein intake per gallon of milk. In this experiment even the control cow showed a small negative balance of -3.7 g N/day in period 2. But, whereas the nitrogen balance of this animal remained almost unchanged in period 3, those of cows 4 and 6 became strongly positive ($+20.9$ and $+21.6$ g N/day), after thyroxine treatment had been discontinued. These positive balances in the last period indicate that the retention of nitrogen after thyroxine treatment was much faster than the loss of nitrogen during treatment. In considering the possible effect of the hormone on the cows' health, it is therefore reasonable to suppose that any ill effects due to increased catabolism during hormone treatment may be offset

Table 5 *A summary of the results required to obtain the nitrogen balances*

(Thyroxine was given during period 2 to all cows except the controls. Results are given in g N/day.)

Exp. no.	Cow no.	Period	Intake*	Output of N in			Total output	Balance
				Milk	Urine	Faeces		
1	1	1	153.4	42.3	31.9	60.4	143.6	+ 9.8
		2	129.1	30.3	32.8	63.5	132.6	- 3.5
		3	126.7	23.2	32.4	54.3	109.9	+16.8
	2 (control)	1	101.1	50.2	29.0	69.4	148.6	+12.5
		2	146.7	44.4	35.1	58.5	138.0	+ 8.7
		3	144.3	41.7	40.2	53.2	135.1	+ 9.2
	3	1	157.2	43.7	25.4	63.1	132.2	+25.0
		2	138.1	50.5	32.9	66.1	149.5	-11.4
		3	135.7	29.6	35.4	56.7	121.7	+14.0
2	4	1	166.3	45.6	40.3	64.8	150.7	+15.6
		2	157.7	41.3	42.0	78.8	162.1	- 4.4
		3	151.9	24.8	37.1	69.1	131.0	+20.9
	5 (control)	1	174.0	45.8	55.7	68.8	170.3	+ 3.7
		2	153.4	39.1	48.4	69.6	157.1	- 3.7
		3	146.2	37.6	44.5	67.3	149.4	- 3.2
	6	1	190.0	50.7	58.4	70.7	188.8	+ 1.2
		2	181.2	55.4	55.7	79.7	190.8	- 9.6
		3	176.4	34.7	40.4	70.7	154.8	+21.6
3	7	1	277.2	74.4	71.9	112.0	258.3	+18.9
		2	315.7	93.0	95.9	120.6	309.5	+ 6.2
		3	250.5	50.4	64.5	108.4	229.3	+21.2
	8	1	256.7	66.6	92.3	93.3	252.2	+ 4.5
		2	261.1	64.2	86.6	102.3	253.1	+ 8.0
		3	172.6	30.6	58.3	79.5	168.4	+ 4.2

* Corrected for food refusals which occurred but seldom and were very small.

In Exp 1, during the thyroxine period, the nitrogen balances for both the treated cows became negative, whereas the nitrogen balance in the control cow remained positive. Of the two treated

by the raised anabolism after the treatment is discontinued.

In Exp 3, in which the allowance of protein per gallon of milk was further increased, the two cows

7 and 8 showed increases in the yields of milk and milk fat which were very similar to those of cow 6 in Exp 2, and yet their nitrogen balances remained positive throughout the entire experiment. The positive balance for cow 7 was reduced during thyroxine treatment, but increased again after treatment ceased. But with cow 8, which showed a much larger stimulation of milk production than any of the other cows, the positive nitrogen balance was slightly increased during hormone treatment. It may be concluded from these findings that, on normal rations, the increased milk yield produced by 10 mg thyroxine/day is accompanied by an accelerated protein catabolism, but that this excessive catabolism may be inhibited by increasing the intake of food.

Partition of urinary nitrogen

(1) *Urea plus ammonia* The proportion of urinary nitrogen excreted as urea plus ammonia was determined for the composite 2 day samples of urine collected from all six cows during the first two experiments. The results showed a constant ratio (60%) of urea plus ammonia to total nitrogen throughout all three periods.

(2) *Creatine and creatinine* The results for the excretion of creatine and creatinine in Exp 2 are shown in Fig 3. The corresponding graphs for the first and third experiments were very similar. No significant change was noted in the creatinine excretion for any of the cows, nor in the creatine excretion of the control animals (e.g. cow 5, Fig 3). With the treated animals, however, changes in the creatine excretion tended to follow changes in milk yield. Thus, in the thyroxine period, the creatine excretion was first enhanced, and then began to fall some time before thyroxine treatment ceased. After treatment ceased creatine excretion fell rapidly to a very low value, the fall corresponding roughly to that in milk yield. Like the milk yield, the creatine output then increased again. In the third experiment, where the rations were much more liberal and where the nitrogen balance was always positive, the creatine excretion was again maximal during thyroxine treatment and minimal immediately after the treatment ceased, but the changes were smaller with these two cows (particularly with cow 8) than with the animals in the first two experiments in which the nitrogen balances became negative during treatment. If it is supposed that the creatine is of catabolic origin, these variations in the amount excreted are just what would be expected. Stimulation of milk yield might well be associated with increased catabolism, and thus would be accompanied by an increased output of creatine. It does not follow, however, that creatine excretion is causally related to milk production, for it is probable that the creatinuria occurring during hormone treatment is

merely a result of the general increase in metabolic rate which thyroxine produces in the tissues as a whole.

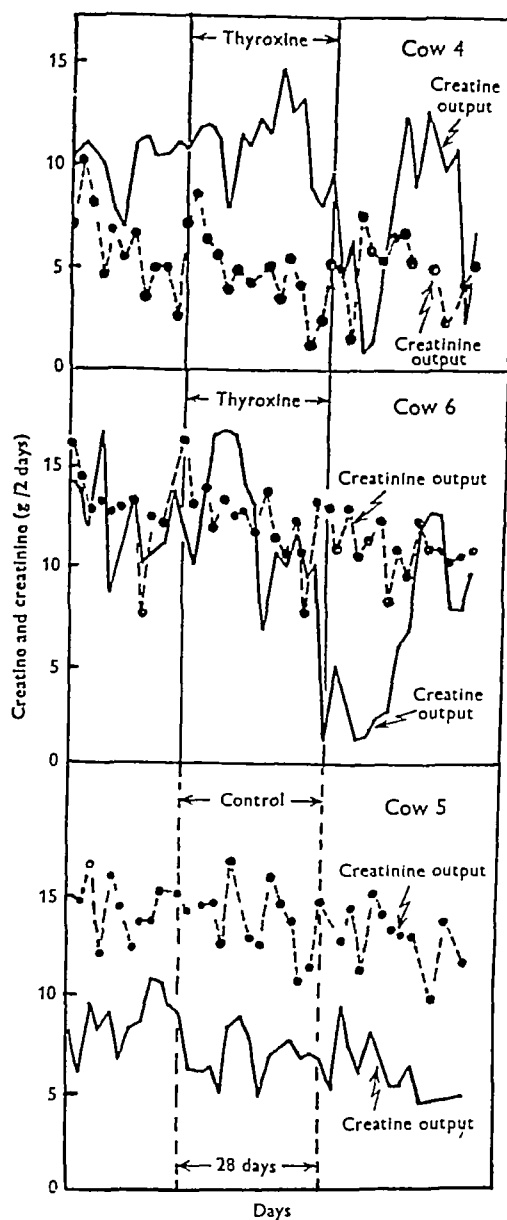


Fig 3 The effect of thyroxine on the output of creatine and creatinine in the urine

For ease in comparing the excretion of creatine and creatinine nitrogen, the results have been recorded in Table 6 as percentages of the total urinary nitrogen. The creatinine figures were variable and showed no consistent change which could be related to the hormone treatment. As would be expected from the creatine outputs (Fig 3), and from the urinary nitrogen outputs (Table 5), the creatine figures for the treated animals tended to

be slightly higher during hormone treatment than before it. When thyroxine administration was discontinued, creatine output decreased. This was marked with cows 1, 3, 4 and 6, but not so marked with cows 7 and 8. The same tendency was noted for control cow 2, but not for control cow 5. This

control cow 2 there was a reasonably close parallelism between the volume and the amount of urinary nitrogen throughout the pre thyroxine and thyroxine periods. This parallelism also existed with cows 1 and 3 before hormone treatment, but, some days after treatment began, a substantial increase

Table 6 Output of creatine and creatinine (expressed as percentage of total urinary nitrogen)

Exp. no	Cow no	Creatine Period			Creatinine Period		
		1	2	3	1	2	3
1	1	9.5	10.5	6.6	4.0	4.4	3.5
	2 (control)	6.1	6.7	5.5	6.4	6.4	5.1
	3	6.8	8.8	4.6	11.1	8.2	7.2
2	4	7.1	7.6	5.4	4.2	3.1	3.9
	5 (control)	4.4	4.1	4.4	7.1	8.0	7.8
	6	5.9	5.8	3.7	6.2	6.1	6.2
3	7	2.3	2.4	2.0	2.9	3.3	3.6
	8	1.0	1.5	1.1	3.4	4.5	5.1

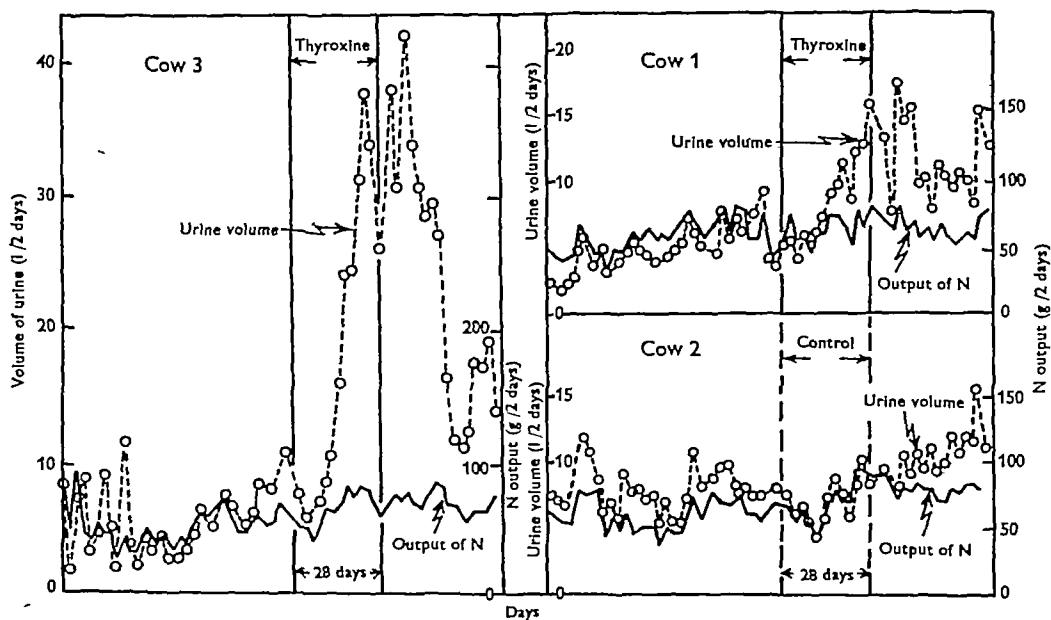


Fig 4 The effect of thyroxine on the volume of urine and on the output of total nitrogen in the urine

difference between the control cows may be related to the fact that cow 5 was fed at a slightly higher level than cow 2. For the best fed cows (7 and 8) the percentage of total urinary nitrogen excreted, as creatine and creatinine, was much smaller than for the other cows. This was due to the fact that the total amount of nitrogen excreted in the urine was naturally higher for the best fed animals than for the others (Table 5).

The diuretic effect

The volume of urine and the urinary nitrogen excreted per day by the cows in the first experiment are shown in Fig 4. It will be noted that for the

in volume occurred. An increase in volume was shown at this stage by all the experimental cows, but not by either of the controls. This may have been caused in part by the cows using water to disperse some of the extra heat generated in their tissues when thyroxine increased the metabolic rate, or it may be that thyroxine raised the activity of the kidneys either directly or by stimulating the secretion of a diuretic hormone. The very large increase in urinary volume noted for cow 3 (Fig 4) was exceptional, the increase in volume with the other experimental animals being of the order of that shown for cow 1. It is not known why the increase with cow 3 was so large.

The effect of thyroxine on the utilization of food

Apparent digestibility of dietary nitrogen An attempt is made to assess the effect of thyroxine on the utilization of food by calculating the average values for apparent digestibility of the dietary nitrogen and the efficiency of its conversion to milk nitrogen in each of the three periods (Table 7). In

Conversion of dietary nitrogen to milk nitrogen The results in Table 7 show that in the first two experiments thyroxine caused a marked increase in the apparent efficiency with which the dietary nitrogen was converted to milk nitrogen. When hormone treatment ceased an even more marked change, in this case a decrease in apparent efficiency, occurred. Of the two control cows (2 and 5) one behaved

Table 7 *The apparent digestibility of the dietary nitrogen and the efficiency of conversion of the dietary nitrogen to milk nitrogen*

(The apparent digestibility is defined as $100 \frac{(\text{Intake N} - \text{faecal N})}{\text{Intake N}}$, the conversion of dietary N to milk N is expressed as the ratio $\frac{\text{Milk N} \times 100}{\text{Intake N} - \text{faecal N}}$)

Exp. no.	Cow no.	Apparent digestibility Period			Conversion of dietary N to milk N Period		
		1	2	3	1	2	3
1	1	55	51	57	50	55	32
	2 (control)	57	60	63	65	50	46
	3	60	52	58	46	71	38
2	4	61	50	55	45	52	30
	5 (control)	61	55	54	44	47	48
	6	63	56	60	50	55	33
3	7	59	62	57	51	50	47
	8	64	60	54	39	40	33

Exp. 1 the apparent digestibility with control cow 2 increased as the experiment progressed, whereas with the treated animals there was a substantial decrease during thyroxine treatment. In Exp. 2 the control animal (no. 5) showed a progressive decrease in the apparent digestibility of its dietary nitrogen. The treated animals, on the other hand, like the treated animals in the first experiment, showed a decrease during hormone treatment, which changed to an increase when hormone treatment ceased. In Exp. 3 there was no significant difference in the results for periods 1 and 2, but when thyroxine treatment ceased the apparent digestibility decreased. This difference between the changes noted in the first two experiments and those in the third is probably associated with the much higher level of feeding in the last experiment. It may be concluded that at the lower levels of feeding the apparent digestibility of the dietary nitrogen tends to be decreased when thyroxine is administered. Estimation of the moisture content of the faeces throughout the entire experiment showed that during thyroxine treatment the faeces were more moist. They were also obviously softer and more liquid than in the control periods. This might tend to cause the contents of the alimentary tract to be retained for a shorter time during thyroxine treatment than is usually the case, and this, in turn, might cause a reduction in the amount of nitrogen absorbed. With cows 7 and 8 the more rapid passage of food caused by thyroxine treatment would tend to offset the effect of the more liberal feeding.

differently from the other in this respect. The efficiency of conversion decreased with cow 2 and increased with cow 5 as the experiment progressed, but they both differed from the experimental animals in not showing a maximum in period 2. In Exp. 3 the better fed cows (7 and 8) did not show any significant change in efficiency of conversion when thyroxine was given, in spite of the larger increases in milk yield. They also showed smaller decreases in efficiency of conversion when thyroxine was discontinued.

DISCUSSION

The experiments reported show that, under conditions of controlled food intake, the effect of thyroxine in stimulating the yield of milk can be attributed mainly to an increase in catabolism. The metabolic rate, as measured by the pulse rate, is much accelerated, so that weight is lost and nitrogen balance becomes negative. However, negative nitrogen balances during thyroxine treatment can be inhibited by a more liberal allowance of food. This diminution of catabolism as a result of increasing food intake recalls a similar phenomenon, reported by Cuthbertson (1942), Croft & Peters (1945) and Taylor (1943), who found that large negative nitrogen balances resulting from trauma could be reversed by increasing the food intake.

The effects of thyroxine in increasing catabolism were reversed during the period following its discontinuance. Nitrogen retention increased markedly, the pulse rate reverted to somewhat less than its

pretreatment level, and the graphs for the yields of milk and milk fat showed distinct minima. During this last period of the experiments tissue anabolism evidently took precedence over milk secretion in its claim for available nutrients.

The foodstuffs used in these experiments were, with the exception of groundnut, such as can readily be grown in Britain. It might, therefore, at times be economic to obtain a temporary increase of the milk supply by the feeding of iodinated casein, the action of which is very similar to that of thyroxine. Although such feeding would temporarily increase catabolism, any consequent ill effects would be largely overcome by the stimulus to anabolism which occurs when the treatment is discontinued. Any subsequent loss of efficiency by the cow would have to be balanced against the temporary gain of milk yield without any increase in the number of cows in the herd, in the man hours or in the materials other than foodstuffs required for its production.

SUMMARY.

1 The effect of injecting 10 mg thyroxine/day for 4 weeks into cows at three different levels of food intake has been investigated. The resultant increase in milk yield was accompanied by an increase in pulse rate, by loss of weight, by markedly negative nitrogen balance and by an increase in the excretion of water and creatine in the urine. The percentage of fat in the milk was increased and small but statistically significant increases were observed in the percentage of solids and of nitrogen in the fat free milk.

2 Thyroxine appeared to stimulate lactation by increasing the cows' catabolism, and this resulted in negative nitrogen balances.

3 The negative nitrogen balances could be inhibited by increasing the intake of food.

The author wishes to thank members of the staff of the Hannah Institute for help in these experiments. Thanks are particularly due to Dr N C Wright for suggesting the investigation, and to Dr J A B Smith for his collaboration.

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The Effect of Thyroxine on the Metabolism of Lactating Cows

2 CALCIUM AND PHOSPHORUS METABOLISM

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In the previous paper (Owen, 1948), experiments have been described on the effect of thyroxine injections on the nitrogen metabolism of lactating cows. During these experiments, data for the calculation of calcium balances were obtained for all the cows, and data for phosphorus balances for two of them. The purpose of the present communication is to report the effect of thyroxine on the calcium and phosphorus contents of the milk, and also on the

calcium and phosphorus metabolism of the cows when thyroxine was administered with the object of increasing the milk yield.

EXPERIMENTAL

The general plan of the experiments and the nature of the rations have been described already (Owen, 1948). There were three experiments, each comprising three periods. The intention was to have three cows in each experiment, but in

the last experiment only two of the three completed the required period in the metabolism house. There were, therefore, eight animals in all. In the first and third periods of each experiment, no thyroxine was given, but in the second period two of the cows were injected with 10 mg thyroxine daily for at least 28 days. The third cow acted as a control and received no thyroxine in any of the periods. For all eight animals Ca was determined in the food, milk, urine and faeces, and from these data the Ca balances were calculated for each period. For the two animals in the third experiment the same was done for P.

Methods Owing to the amount of work involved in metabolism studies of this type with large animals, the Ca and P analyses had to be carried out after the main experimental work was finished. It was necessary, therefore, to store samples of the food, milk, urine and faeces. Milk samples were preserved with formaldehyde, faeces were

RESULTS

Milk composition The average figures for the content of calcium and phosphorus in the fat free milk are shown in Table 1. Inspection and statistical analysis of these results showed that the variations which occurred in the calcium content could not be attributed to the administration of thyroxine. Thus, with cows 1, 3, 6 and 7, there was a slight increase in the calcium content during thyroxine treatment as compared with the preceding period, whereas, with cows 4 and 8, there was a slight decrease. Of the control cows, no. 2 showed a slight decrease and no. 5 a slight increase in calcium content during period 2 as compared with period 1.

Table 1 *The calcium and phosphorus contents of the fat free milk before, during and after thyroxine treatment*

(Thyroxine was given during period 2, except to the control cows 2 and 5)

Exp no	Cow no	Calcium (g/100 ml)			Phosphorus (g/100 ml)		
		Period			Period		
		1	2	3	1	2	3
1	1	0.104	0.115	0.130	—	—	—
	2 (control)	0.114	0.109	0.127	—	—	—
	3	0.107	0.115	0.117	—	—	—
2	4	0.125	0.119	0.125	—	—	—
	5 (control)	0.090	0.094	0.095	—	—	—
	6	0.130	0.138	0.142	—	—	—
3	7	0.128	0.142	0.130	0.0945	0.1089	0.0966
	8	0.141	0.133	0.134	0.0946	0.1106	0.1008

stored dry in stoppered bottles and urine samples were kept in a refrigerator. Before analysis the urine samples were made acid to dimethylaminoazobenzene (Töpfer's reagent, pH 2.9) by additions of 6N HCl. By this means any deposits of Ca and phosphate were dissolved, and copious precipitation of uric acid occurred. Analysis of this precipitate, however, showed that it did not contain any Ca or P. Every second 2 day sample of food, milk and excreta was analyzed.

Analytical methods used were identical with those employed previously in human balance experiments (Owen, 1939). The samples were ashed in vitreous basins in an electric muffle furnace which was thermostatically controlled so that its temperature did not exceed 600°. 10% Mg(NO₃)₂ was added to food, milk and faeces to prevent loss of P by volatilization, and the samples were dried on a sand bath before being placed in the muffle furnace. The ash was dissolved in warm 6N HCl and made up to a known volume. After silica had been allowed to settle overnight, samples were taken for analysis. With urine it was found impracticable to estimate Ca and P on the same sample of ash. This was because, on using Mg(NO₃)₂ to bind the P, serious deflagration occurred when the mixture was dried, whilst with MgCl₂, the resulting glassy ash caused the basins to crack on cooling. For estimating P, therefore, the urine was digested with H₂SO₄ and 30% H₂O₂ (Horecker, Ma & Haas, 1940), and for the estimation of Ca the urine was ashed in the muffle furnace without addition of magnesium salts. The final stage in the estimation of calcium was the titration of Ca oxalate with KMnO₄. In the P estimation the strengths of the coloured solutions were determined in a Spekker absorptiometer.

The phosphorus results were obtained for two cows only, but with both of them, there was a marked increase in the phosphorus content of the fat free milk during thyroxine treatment, and after treatment ceased the values tended to return towards their pre thyroxine level. The increases of 15 and 17%, which occurred during hormone treatment as compared with the pre thyroxine level, were statistically significant.

Calcium metabolism During all periods of the experiments (Table 2), the cows were in negative calcium balance, except for cow 4 which showed a small positive balance of 0.9 g Ca/day in the period after thyroxine treatment ceased. The type of fluctuation occurring from one 4 day period to another, as found in Exp. 3, can be seen from Fig. 1. It is clear that the output of calcium in the urine did not vary appreciably, and that it was not affected by thyroxine treatment.

It will be noted that a minimum occurred in the graph of calcium intake (Fig. 1). This resulted from the fact that the chief source of calcium in the diet was the hay, and during that particular period of Exp. 3 the calcium content of the hay was low. The apparent digestibility of the calcium is shown in Table 2. The values became negative in periods 2 and 3 of the third experiment.

Table 2 *The apparent digestibility of calcium and a summary of the results required to obtain the calcium balances*

(Thyroxine was given during period 2, except to the control cows 2 and 5 All results which are average figures are given in g Ca/day)

Exp no	Cow no	Period	Ca intake	Output of Ca			Total output	Balance	Apparent* digestibility of Ca
				Milk	Urine	Faeces			
1	1	1	14.6	9.6	0.6	11.1	21.3	- 6.7	24.1
		2	14.6	8.3	0.4	12.5	21.2	- 6.4	15.0
		3	14.6	5.1	0.9	11.9	17.9	- 3.3	19.0
	2 (control)	1	15.2	13.2	0.9	11.0	25.1	- 9.9	27.8
		2	15.2	11.8	0.8	9.1	21.7	- 6.5	40.5
		3	15.2	11.5	0.6	9.8	21.9	- 6.7	35.6
	3	1	14.9	12.4	1.2	9.4	23.0	- 8.1	37.2
		2	14.9	13.1	1.8	9.1	24.0	- 9.1	38.9
		3	14.9	7.3	2.6	7.3	17.2	- 2.3	51.0
2	4	1	18.3	10.4	0.7	12.8	23.9	- 5.6	30.4
		2	19.3	10.0	1.1	16.0	27.1	- 7.8	16.9
		3	19.2	5.5	1.2	11.6	18.3	+ 0.9	39.8
	5 (control)	1	19.6	8.9	1.5	12.7	23.1	- 3.5	34.8
		2	19.1	7.6	2.0	14.4	24.0	- 4.9	24.6
		3	18.9	7.4	2.0	13.9	23.3	- 4.4	26.8
	6	1	20.3	14.8	1.5	13.5	29.8	- 9.5	33.5
		2	20.3	15.4	0.7	14.2	30.3	- 10.0	29.7
		3	20.2	8.2	1.2	13.2	22.6	- 2.4	35.0
3	7	1	41.0	21.6	1.8	33.8	57.2	- 16.2	17.2
		2	40.0	26.8	1.5	40.9	69.2	- 29.1	- 2.2
		3	31.4	15.1	1.5	36.8	53.4	- 22.0	- 17.2
	8	1	39.4	20.6	1.8	31.4	53.8	- 14.4	20.3
		2	36.2	18.3	1.2	38.7	58.2	- 22.0	- 6.9
		3	25.6	7.5	1.7	35.1	44.3	- 18.7	- 37.1

$$* 100 \frac{(\text{Intake of Ca} - \text{faecal Ca})}{\text{Intake of Ca}}$$

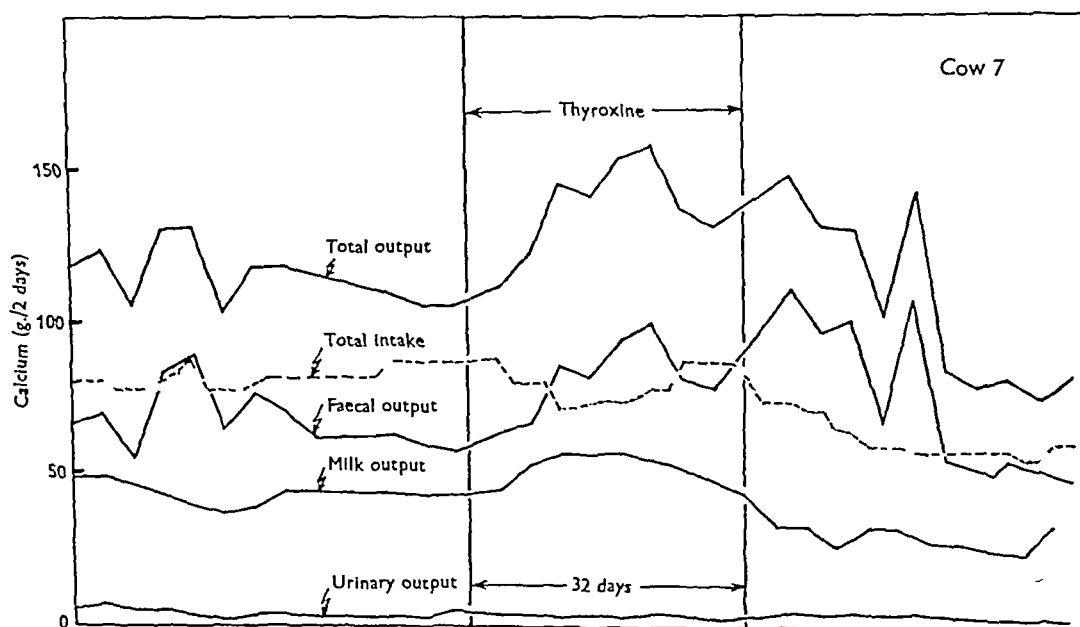


Fig 1 The effect of thyroxine on the output of calcium in milk, urine and faeces The calcium intake is also shown

Phosphorus metabolism The data obtained for calculating the phosphorus balances for cows 7 and 8 are shown in Table 3. The balances were all positive throughout the whole of Exp. 3, and in this respect they resembled the nitrogen balances rather than the calcium balances.

attributed largely to the decrease in milk yield, which resulted in a diminished output of calcium in the milk during the third period. In the third experiment, the negative balances were more marked during thyroxine treatment than either before or after treatment. This was due mainly to an increase

Table 3 *The apparent digestibility of phosphorus and a summary of the results required to obtain the phosphorus balances in Exp. 3*

(Thyroxine was given during period 2. All results which are average figures are given in g. P/day.)

Cow no	Period	Intake of P	Output of P			Total output	Balance	Apparent* digestibility of P
			Milk	Urine	Faeces			
7	1	51.3	15.8	0.25	30.5	46.6	+ 4.7	40.6
	2	66.2	20.1	0.62	35.1	56.1	+10.1	46.6
	3	51.2	11.1	0.41	31.6	43.1	+ 8.1	38.3
8	1	45.7	14.2	0.18	27.3	41.7	+ 4.0	40.3
	2	55.0	15.0	0.78	29.3	44.9	+10.1	46.7
	3	35.9	5.8	0.44	23.4	29.6	+ 6.3	34.8

$$* 100 \frac{(\text{Intake of P} - \text{faecal P})}{\text{Intake of P}}$$

The outputs of phosphorus in the milk, faeces and urine were all greater in the thyroxine period than in the periods preceding and following hormone treatment. Unlike the corresponding results for calcium, the urinary output of phosphorus was very markedly increased, but this made little difference to the total excretion since the proportion of the output accountable to the urine was very small. In spite of the fact that the output of phosphorus was increased during the thyroxine period, the balances showed that the amount of phosphorus retained by the cows was also greatest in that period. It was possible to have increases occurring simultaneously in both output and retention in period 2, since during that period extra rations were fed according to the increase in milk production, and this led to a greater intake of phosphorus. The apparent digestibility of the phosphorus (Table 3), unlike that of calcium (Table 2), was higher during the thyroxine period than in the other two periods.

DISCUSSION

Negative calcium balances in lactating animals in early or mid-lactation are quite commonly observed (Mackintosh, 1938; Garry & Wood, 1946). It is not surprising to find, therefore, that with the animals used in the present work, the calcium balances were almost all negative, the only exception being the value for cow 4 in the third period of Exp. 2 which was slightly positive. In the first two experiments the balance in the first period was on the whole very similar to that during thyroxine treatment, but a big decrease in negative balance occurred after thyroxine administration had ceased. This can be

in the output of faecal calcium during the thyroxine period. Indeed, it can be seen from Fig. 1 that faecal output of calcium was closely paralleled by total output. Before these results were obtained, it was expected, from the work of Hunter (1930) on humans, that thyroxine treatment would cause all the cows to exhibit a marked increase in the amount of calcium excreted in the urine. This, however, did not occur. Owing to the bulkiness and relative indigestibility of their food as compared with that of non-vegetarian animals, cows void more water in their faeces than in their urine, and also, their urine tends to be more alkaline. Possibly it is due to both these facts that the faecal but not the urinary excretions of calcium were increased. The absence of effect of thyroxine on the urinary excretion of calcium, and its effect on the faecal calcium, is well shown with cow 7 in Fig. 1. The corresponding graphs for cow 8 were very similar. The only cow which showed an increased output of urinary calcium during thyroxine treatment, the increase continuing after thyroxine injections ceased, was cow 3, and it was this cow which excreted an exceptionally large volume of urine in the latter part of period 2 and the early part of period 3 (Owen, 1948).

The results for the apparent digestibility of the dietary calcium, which are shown in Table 2, are very low. Indeed with cows 7 and 8, negative values were obtained in the second and third periods of Exp. 3, more calcium having been voided in the faeces than had been taken in in the food. Hunter (1930) records that Aub observed this same phenomenon in humans. In these two cows, therefore, thyroxine must have brought about a large increase in the production of faecal metabolic calcium, and

even after thyroxine treatment had ceased, the excretion of calcium relative to the amount taken in still remained very high

These negative calcium balances raise the question as to whether cows on winter rations, to whom thyroxine is being administered either as the hormone itself or as iodinated casein, should be given some additional source of readily assimilable calcium. Even if negative calcium balances could not be entirely prevented by dietary measures, it is reasonable to expect that extra dietary calcium would help to replace the loss engendered by increased excretion in the faeces. If this extra loss of calcium in cows treated with thyroxine were not replaced, the cows might have to start their next lactation with depleted stores of mobilizable calcium. Progressive chronic depletion of the skeleton by successive reproductive cycles of pregnancy and lactation on diets containing suboptimal amounts of calcium has been recorded in so many species of animals by many observers, that an extra drain caused by the administration of thyroxine should not be allowed to go uncompensated. Some source of extra readily assimilable calcium such as steamed bone flour or defluorinated rock phosphate, or fish meal, could with advantage be included in the rations of any cow to which iodinated casein or other source of exogenous thyroxine is to be fed.

Since some 99 % of the calcium and some 70–80 % of phosphorus in the animal body are in the skeleton (McCollum, Orent Keiles & Day, 1939), it is perhaps surprising to find that the phosphorus metabolism was so different from that of the calcium. The phosphorus balances in Table 3 were all positive, and they were more strongly positive in the thyroxine period than in either of the other two periods. Unlike that of the calcium, the apparent digestibility of the

phosphorus was increased during thyroxine treatment.

It would appear from these results that phosphorus deficiency is not likely to occur in cows which are receiving thyroxine or iodinated casein, but this conclusion can be put forward only tentatively since it is realized that the results on which it is based were obtained with only two cows.

SUMMARY

1 Eight Ayrshire cows in mid-lactation were used to determine the effect of thyroxine administration on the metabolism of calcium and phosphorus. With one exception, the calcium balances were negative both for the treated and for the control animals throughout all the experiments.

2 With two of the cows which were fed more liberally than the others, the output of calcium was greatest in the thyroxine period, and this was attributable to an increase in the faecal metabolic calcium resulting from the hormone treatment. There was no increased output of calcium in the urine.

3 The apparent digestibility of the dietary calcium was found to be very low. Thus, together with the negative balances, might well result in thyroxine treatment leading to a depletion of calcium reserves which would be dangerously excessive unless dietary measures were taken to make good the loss.

4 Unlike those of calcium, the phosphorus balances (obtained for two cows only) were positive throughout the whole experiment, thyroxine actually tending to increase the amount of phosphorus retained.

5 The calcium content of the milk was not affected by thyroxine, but the phosphorus content was significantly increased.

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Lignins in Young Plants

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In contrast to the extensive research into the properties and structure of the wood lignins, little work has been done on the lignins which occur in the cell walls of annual plants in considerable quantities. These amount to as much as 10–20% of the dry matter in the common forage plants, according to the analytical methods usually employed, which use concentrated mineral acids for lignin determinations. Degradation experiments have been carried out only with lignins contained in the more woody tissues of this kind, such as maize cobs or various straws, where a closer similarity to the wood lignins exists (Phillips, 1944). The lignins of young green plants seem to be digestible to a certain extent (Juon, 1934, Crampton, 1939, McAnally, 1942, Bondi & Meyer, 1943), whereas lignins in straw or maize cobs were found to be practically indigestible (König & Furstenberg, 1906–7, Thomann, 1921, Rogozinski & Starzewska, 1929, Ferguson, 1942).

Lignins from young plants and from animal faeces have, to our knowledge, not been prepared in pure form. This paper presents an attempt to isolate lignins in a state of comparative purity from a number of young plants, and to investigate their chemical properties in order to compare them with the better known wood lignins. Furthermore, lignins were prepared from the faeces of sheep which had been given the same plants as their sole food. This procedure allowed us to follow the chemical changes undergone by the lignins in the body of a typical ruminant.

EXPERIMENTAL

Materials

All the plants used for lignin extraction (and fed to sheep for obtaining faeces) were annuals, as follows, the age at cutting being given in parentheses:

Eragrostis tef (25 months), *Pennisetum* (25 months), *Setaria italica* (25 months), and *Hordeum murinum*, wild barley (35 months) represent the Gramineae.

Trifolium alexandrinum bersem, Bersem clover (15 months), *T. alexandrinum* fahl, Fahl clover (25 months), *Arachis hypogaea*, peanut hay (4 months), and *Lathyrus ochrus*, Cyprus vetch (2 months) represent the Leguminosae.

Plants belonging to these two families were chosen because they show characteristic differences. Leguminosae remain comparatively soft and non-ligneous until the last stages of their development, while in the Gramineae woodiness sets in early especially in the stalks. None of the plants used in this investigation was very woody. Peanut hay and wild

barley were the materials in which woodiness had developed most.

The plant material was divided into two parts: one was dried and the lignin subsequently extracted, the other part was fed to sheep. In accordance with the usual procedure in digestibility experiments (see Bondi & Meyer, 1940), the various plants were in each case given to the sheep, kept in metabolism cages, as their only food. After a preliminary period of 6 days, faeces of the sheep were collected during the following experimental period of 10 days. From the dried sheep faeces, lignin was extracted as in the case of the plants.

Extraction methods

The separation of lignin from the other components of the plant can be achieved in two ways. One is to dissolve out all materials other than lignin, and leave it as the only remaining material. In the second method the lignin is dissolved out of the plant tissues leaving the other materials. The first method requires the action of concentrated mineral acids on the plant material. In our experience, however, this method is not suitable for the preparation of larger quantities of lignin from green plants, since the action of strong acids may cause the condensation of soluble carbohydrates contained in them, giving black insoluble substances often erroneously classified as 'lignins' (see Hilpert & Hellwage, 1935).

The second type of methods for the isolation of the lignin includes those in which lignin is extracted by the action of organic solvents such as ethanolic HCl or phenol, or by alkali. We did not use organic solvents for extraction, since these bring about condensation reactions or even degradation of the lignin. Instead, we had recourse to the extraction of lignin by means of alkali. At first we used the method proposed by Phillips & Goss (1934) for the extraction of lignin from maize cobs. They extracted the lignin with 60% ethanol containing NaOH (2% w/w) and, after evaporating the ethanol, precipitated it with an excess of ice-cold 0.4N-H₂SO₄. The yields of lignins obtained from green plants by these methods were very small: 0.8–1.6% of lignin were obtained from plants, and 0.9–1.8% from faeces. These figures are much smaller than those obtained in the analytical determination of lignin by treatment with concentrated acids, which gives values of 10–15% in the same forage plants and faecal matter. The lignin obtained by Phillips's method also contains 1.1–3.2% N, according to the type of forage plant. Neither an increase of the

concentration of the ethanol in the extracting solution (up to 96 %), nor an increase in the concentration of NaOH (up to 10 %), brought about either a noteworthy increase in the lignin yield or a decrease in the nitrogen content of the lignin. The raising of the ethanol concentration in the extraction liquid brought about a decrease in the lignin yield.

Since, in spite of the varying of the experimental conditions, the extraction of lignin with ethanolic NaOH did not give practical yields, another method was worked out using hot aqueous 0.5N NaOH (see also Phillips, 1929). By this method lignins are extracted together with considerable amounts of polysaccharides, probably hemicelluloses. Both materials are precipitated by acidifying the alkaline extract. The lignins are separated from the hemicellulose by redissolving the precipitate in alkali and adding a large excess of slightly acidified ethanol, which dissolves the lignins, leaving the hemicellulose. The ethanol soluble lignins still contain about 20 % of hemicellulose, which can, however, be removed easily by a mild acid hydrolysis. The experimental details are as follows.

The plant or faecal material is ground coarsely, extracted thoroughly with ether and dried. 1 kg. of this material and 12 l. of 0.5N NaOH are put in a vessel of about 16 l. capacity (to avoid foaming over), and heated for 8 hr. to 80–85°, with continuous stirring. The entire contents of the vessel are filtered through cheese cloth, and the residue is washed several times with water (5–6 l. in all). The washings are combined with the filtrate, while the residue is extracted three more times under the same conditions. The extracts are combined and evaporated *in vacuo* to about one third of their original volume. After cooling, the residue is acidified with 5N H₂SO₄. This precipitates the lignin (together with considerable amounts of hemicellulose) which is allowed

to settle out, after which the supernatant liquid is decanted. The precipitate is washed several times with water, which is decanted off each time, until the supernatant liquid appears only slightly yellow. The precipitate is then separated by centrifuging at 2000 r.p.m. for 15 min., or by filtration.

In order to purify the crude product it is again dissolved in the smallest possible quantity of warm aqueous 1.25N NaOH. This solution is added to 4 vol. of 96 % ethanol and acidified slightly. The dark brown supernatant liquid which contains the lignin is separated from the residue (1) by filtration or centrifuging. The greater part of the ethanol is then driven off, this causes precipitation of the lignin. The resulting aqueous suspension of lignin is treated with enough NaOH to dissolve all the lignin, and then 5N H₂SO₄ is added to make it just acid, causing separation of the lignin in voluminous flakes. The lignin is difficult to filter and contains varying amounts of hemicellulose. In order to remove the latter, the acidified suspension is heated to boiling. At this temperature hydrolysis of the hemicellulose and coagulation of the lignin take place, and the lignin precipitate becomes coarse and easily filterable. The impurities remain in the solution, which is usually deep brown in colour. During the first precipitation the lignin loses 12–15 % of its weight. A second precipitation causes it to lose 2–3 %; in subsequent reprecipitation no measurable quantities of lignin are lost. After the second reprecipitation, the N, ash, and OCH₃ contents of the lignin remain constant. Lignin obtained in this manner still contains 1–2 % of ash.

The residue (1) remaining after solution of the lignin is again dissolved in warm 1.25N NaOH. The solution is filtered or centrifuged, acidified with 5N H₂SO₄, and mixed with 5 vol. of 96 % ethanol, whereupon precipitation of yellow flakes takes place and the lignin still present dissolves in the ethanol. Solution and reprecipitation are repeated, until the supernatant fluid is colourless. The ethanolic solutions are collected and treated for a further yield of lignin. The precipitated flakes are suspended in water and washed by decantation till the complete disappearance of SO₄²⁻. The lignin fraction obtained by separation from the

Table 1 Yields of lignin and nitrogen contents of the different lignins and the original materials

Material		Yields from dried original material (%)	N contents	
			Lignin (%)	Dried original material (%)
<i>Fragrostis tef</i>	Plant	5.4	1.18	1.86
	Faeces	9.0	1.31	1.15
<i>Panicum</i>	Plant	4.6	1.21	1.68
	Faeces	7.1	1.40	1.68
<i>Setaria italica</i>	Plant	5.1	1.41	1.08
	Faeces	6.8	1.62	1.20
Wild barley	Plant	5.1	1.63	0.52
	Faeces	6.5	1.70	0.71
Berseem clover	Plant	5.0	2.92	2.99
	Faeces	4.6	3.17	2.16
Fahli clover	Plant	3.0	3.36	1.67
	Faeces	3.4	3.92	1.52
Peanut hay	Plant	5.2	3.24	3.12
	Faeces	6.3	3.45	3.28
Cyprus vetch	Plant	4.1	3.30	2.48
	Faeces	6.5	3.41	1.94

hemicellulose residue was always identical with the principal product (containing the same percentage of N and OCH_3) and, therefore, it is combined with the latter

The combined precipitates of hemicelluloses are then suspended in acetone, in which they are boiled three times, filtered and dried at 40° *in vacuo*. This material, on hydrolysis with boiling 0.4N H_2SO_4 , left a residue of about 10% consisting of lignin. The rest was split completely into reducing sugars, and is therefore assumed to be a hemicellulose

Yield and N content of the lignins prepared from different plant and faeces materials are presented in Table 1, and their OCH_3 contents in Table 3. In several cases the lignins obtained in the first to fourth extraction were precipitated and treated separately. The N and OCH_3 contents of the lignins prepared from the different extracts were practically uniform, thus eliminating the need for their separate treatment throughout

Properties of lignin

The various lignins prepared by extraction with 0.5N NaOH are light to dark brown, fine powders, decomposing without melting on being heated to 400° . They are easily soluble in dilute NaOH, in ethanol, and in acetone. Up to 70% of the material is soluble in 98% acetic acid. It should, however, be noted that completely dry, glacial acetic acid will not dissolve lignin either cold or hot, solution only occurs after the addition of 2–3% of water. Phenol and β -naphthol dissolve up to 15% of their weight of lignin when heated about 20° above their melting points. Lignins are not soluble in other common solvents

The lignins prepared by us proved to be completely free from carbohydrates. The hydrolysates obtained according to Waksman's method for the determination of hemicellulose and cellulose were always found to be completely free of reducing sugars (cf Bondi & Meyer, 1943). Nor could furfural be obtained by distillation with 3N -HCl, as in the Tollens pentosan determination

Nitrogen content of lignin

Wood lignin is practically free from N. Very small quantities of N (0.2–0.3%), found in wood lignin, are considered by Kalb (1923) and Paloheimo (1925) to be accidental impurities caused by protein materials hydrolyzable only with difficulty, and Waksman (1938) assumes that there are lignin proteimates present in humus lignin which are difficult to split. Phillips, Goss, Davis & Stevens (1939) found some N in crude lignin from oats, which decreased in quantity with increasing age of the plant. Our experiments show with certainty that the N content of lignin prepared from green plants is due neither to accidental contamination with protein nor to the presence of lignin protein complexes. The constancy of the N content of lignin prepared from the same

plant material in consecutive extractions and by different methods shows that there can be no question of fortuitous contaminations

In order to avoid the formation of lignin protein complexes during extraction, an attempt was made to remove the protein before extraction by treatment of the plant material with 0.05N -NaOH. It was found that 80–90% of the protein present in the original material was removed by this preliminary treatment, but that at the same time most of the lignin also went into solution. Lignins prepared from material that had undergone this preliminary treatment were practically equal in N content to those obtained without preliminary treatment. On the other hand, the yield of lignin was greatly reduced, so that this preliminary treatment with NaOH was of no practical use

A trial was also made of treatment of the plant material with a solution of pepsin in dilute HCl in order to secure a diminution of the protein content. By this method, too, 60–75% of the protein present was removed, but the lignin from plants given this preliminary treatment showed approximately the same N content as that from plant material which did not receive it. This equal N content of lignins prepared from protein-rich plants and of those from which most of their protein had been removed, argues against the hypothesis that condensation between lignin and protein takes place during the extraction of lignin

Finally, experiments were undertaken which were intended to find out whether the N in lignin was protein N or not. The N content of the lignins was not diminished by vigorous acid hydrolysis (refluxing for 24 hr with 5N H_2SO_4) or by mild hydrolysis (digestion with 0.5N -HCl for 24 hr at 38°). Both treatments bring about the dissolution of 10–15% of the lignin, but the solutions do not contain N and the N content of the remaining lignin rises proportionally to the weight loss. Repetition of the hydrolytic treatment leaves the lignin completely unchanged. The same result was obtained when pepsin was added to the HCl solution. The N-containing portion of the lignin was equally resistant to pancreatin at its optimum pH of 8.0. At this pH lignin is soluble, but no degradation of the lignin N could be obtained by this method. The resistance of the lignin N to hydrolysis by mineral acids or by enzymes shows that it cannot be protein N. Additional evidence for the non protein nature of lignin N is furnished by the fact that the N content of faecal lignin is not lower than that of plant lignin, i.e. the digestive enzymes of the ruminant have no influence whatsoever on lignin N

Nor can lignin N be primary or secondary amine N, as is shown by the resistance of lignin N to distillation of the lignins with concentrated NaOH and to treatment of the lignins with NaNO_2 (no liberation

of N occurred and the N content of the lignin remained unchanged) Therefore, the only possibility left is that the N is present as tertiary N, possibly as a cyclic compound In this connection it may be mentioned that derivatives of pyridine have been found in humus lignin (see Waksman, 1938), and that pyridine and its homologues appear regularly in the dry distillation of wood

In order to test this assumption of the presence of heterocyclic N compounds in lignin, the following experiments were carried out

According to Shaw (1937) pyridine decomposes on reduction with Na in absolute ethanol to give NH_3 and glutardialdehyde Our lignins were, therefore, also subjected to reduction with Na and ethanol, as well as with Zn dust in alkaline solution These treatments degraded the lignin completely to N free products of low molecular weight, while all the N is driven off as NH_3 during the reaction with Zn dust, and may be collected in acid and determined by back titration of the latter (In one set of experiments, 50–100 hr were necessary for complete liberation of the lignin N, when 5.0 g lignin were refluxed with 60 g Zn dust, 100 g NaOH and 450 ml water)

Similar results were obtained by reducing the lignin with Na in ethanol 10 g lignin were refluxed in 300 ml absolute ethanol, while 50 g Na were added in small portions The reaction was terminated in 30–40 hr, lignin N being recovered as NH_3

Preliminary experiments showed that oxidation of lignin with KMnO_4 in alkaline solution also produced NH_3 These experiments were then repeated quantitatively, in accordance with the method used by Charnbury, Eckert, LaTorre & Kinney (1945) in investigating the nature of the N in humic acids This determination showed that all lignins yield 70–80 % of their N content as NH_3 in the course of

oxidation For comparison, a derivative of pyridine, nicotinic acid, was subjected by us to the same treatment and gave similar results (see Table 2) The similar behaviour of the N contained in nicotinic acid and in our lignins allows us to infer that the N in lignins and in compounds containing tertiary N is bound similarly

Table 2 *Liberation of nitrogen of lignins as ammonia by oxidation with KMnO_4*

Lignin from		Percentage N before oxidation	Loss of N as percentage of total N
<i>Eragrostis tef</i>	Plant	1.18	76.2
	Faeces	1.31	75.0
<i>Pencillaria</i>	Plant	1.21	73.9
	Faeces	1.41	76.2
Wild barley	Plant	1.63	77.8
	Faeces	1.70	78.1
Fahl clover	Plant	3.36	74.6
	Faeces	3.91	75.9
Nicotinic acid		9.44	78.4

Hydroxyl and methoxyl groups in lignin

The OCH_3 content of different wood lignins varies from 14 to 20 %, and is higher in hardwood lignins than in softwood lignins According to Phillips *et al* (1939) and Bondi & Meyer (1943) the OCH_3 content of lignin isolated from green plants is lower than that of wood lignin, and increases with the age of the plant In barley, for instance, the OCH_3 content of the lignin increases during growth from 3 to 10 % (Phillips *et al* 1939)

Table 3 gives the OCH_3 contents of the various lignins isolated by us from plants and faeces These figures fall naturally into two groups lignin from the Gramineae contains 9–10 % OCH_3 , while the lignin of the Leguminosae contains approximately

Table 3 *Methoxyl content of lignins*

Lignin from		Percentage OCH_3 in lignin		
		As prepared	After methylation	
			With CH_3N_2	With $(\text{CH}_3)_2\text{SO}_4$
<i>Eragrostis tef</i>	Plant	9.71	19.00	23.48
	Faeces	9.70	19.02	19.34
<i>Pencillaria</i>	Plant	9.65	18.77	22.75
	Faeces	9.65	19.03	19.10
<i>Setaria italica</i>	Plant	9.60	18.94	23.04
	Faeces	9.70	18.81	18.92
Wild barley	Plant	9.85	19.21	21.33
	Faeces	9.77	18.48	18.59
Bersem clover	Plant	5.10	15.29	18.08
	Faeces	5.22	15.01	15.10
Fahl clover	Plant	5.75	15.31	16.70
	Faeces	6.67	15.42	15.51
Peanut hay	Plant	4.28	13.40	15.20
	Faeces	4.40	14.10	14.09

one half of this amount. It is notable that a material like peanut hay, which on inspection appears much more woody than, say, barley, nevertheless shows clearly by the OCH_3 content of its lignin that it belongs to the Leguminosae, this shows that the family to which the plant belongs has, at least in this case, a stronger effect than the age and degree of 'woodiness'.

Table 3 also shows that the OCH_3 content of the lignin isolated from faeces differs hardly at all from that of the corresponding plant lignins, i.e. there is no noteworthy rupture of ether linkages in the alimentary tract.

The determination of OCH_3 in lignin was supplemented by the estimation of OCH_3 introduced into the lignin. All samples of lignin were methylated by two different methods, with dimethyl sulphate and with diazomethane, according to the procedures given by Phillips (1944).

The results of OCH_3 determinations in the methylated lignins are also given in Table 3. The OCH_3 content of lignins from Leguminosae is trebled on methylation with diazomethane, whilst the content of Gramineae lignins is only approximately doubled. We conclude from this that the OCH_3 groups in lignins from Gramineae (which have the higher OCH_3 content before methylation) correspond to unsubstituted hydroxyl groups in the lignins from Leguminosae. Methylation with dimethyl sulphate brings about only slightly larger increases in OCH_3 content than that obtained with diazomethane. Comparison of the results of methylation with dimethyl sulphate and diazomethane therefore shows that in all plant lignins there are many more phenolic (and enolic) hydroxyl groups than aliphatic ones. Since faeces lignins, unlike plant lignins, gain the same amount of OCH_3 by methylation with dimethyl sulphate and with diazomethane, it seems that in faecal lignins non phenolic hydroxyl groups are completely absent.

Determination of the molecular weight of lignin

The values in the literature of the molecular weight of lignin vary widely. In earlier publications Beckman & Liesche (1921*a, b*) found, by cryoscopic determinations in phenol, values of 762 and 784 for

the molecular weight of lignin prepared from wheat straw, later Fuchs (1936) found a value of 900. More recently, Conner (1941) determined a value of 3900 by dielectric measurements. These figures are comparatively low values for high polymers, but become more plausible when we consider that the properties of lignin are different from those of other high polymer plant materials. The structure of lignin is not fibrous and the viscosity of its solution is low.

Freudenberg (1939) assumes that lignin has a three dimensional structure created by the polymerization of from 2 to 10 molecules of coniferyl alcohol. This would indicate that the molecular weight was comparatively low. Substances like magnalol and larciresinol (the so called lignanes, containing 2 or 3 hydroxyconiferyl alcohol molecules) have an even lower molecular weight (see Haworth, 1942).

The differences between the molecular weight obtained by the cryoscopic method and those obtained by other physical methods appear to be due to the depolymerization of the lignin by the solvents used in the cryoscopic determination, so that the result is the molecular weight, not of the lignin, but of the 'structural units' of which it is composed. Furthermore, the molecular weights given in Table 4 should be regarded as averages only, since there is no evidence that the lignin molecule breaks down to give units identical in molecular weight. The molecular weights given in Table 4 should be accepted with this reservation. It should be noted that the molecular weights of lignins obtained from plants and from faeces are of the same order of magnitude.

The lignins used for cryoscopic determination of their molecular weight were purified by twice dissolving in 98% acetic acid, reprecipitating by dilution with ice water and by drying in a vacuum over solid NaOH at room temperature. β -Naphthol was employed as a solvent, its molecular freezing point depression was determined by Brum (1923) as 11.25°. The determinations were carried out in a conventional Beckmann micro apparatus with an electro magnetic stirring device, glycerol was used in the bath for maintaining temperatures between 115 and 130°.

Table 4 *Molecular weight of lignins determined by depression of freezing point of β naphthol*

Lignin from		Weight of lignin (g)	Weight of β naphthol (g)	Δt	Molecular weight
<i>Pencillaria</i>	Plant	0.1034	2.1272	0.861	635
	Faeces	0.1078	2.2964	0.804	657
Wild barley	Plant	0.1661	1.9470	1.500	640
	Faeces	0.1388	1.8696	1.327	629
Fahl clover	Plant	0.2076	1.7250	2.205	614
	Faeces	0.1240	2.2095	1.042	606
Peanut hay	Plant	0.1002	0.8228	2.203	621
	Faeces	0.1228	1.5180	1.497	608

Degradation of the lignins

A number of degradation experiments were undertaken with the purpose of comparing the degradation products obtained from plant and faeces lignins and from wood lignins. The processes used in this investigation were the following (1) oxidation with nitrobenzene in alkaline solution, (2) fusion with potassium hydroxide, (3) hydrolysis with acid ethanol under pressure.

(1) *Oxidation of lignins with nitrobenzene and alkali* This reaction, which was successfully used by Freudenberg, Lantsch & Engler (1940) and by Creighton, Gibbs & Hibbert (1944), consists in treatment in NaOH solution with nitrobenzene at 160–200° under pressure for several hours. After removing the reduction products of the nitrobenzene by steam distillation the solution is acidified and extracted with ether. The ether solution is successively extracted with solutions of NaHSO₃, NaHCO₃ and NaOH, to separate aldehydes, acids and phenols, respectively. Four 30 g samples of each lignin were treated with nitrobenzene and were combined before the ether extraction. The aldehyde extracts were acidified and SO₂ removed *in vacuo*, after which the solution was divided into two parts. The total aldehydes were precipitated from one part with a 2% solution of 2,4-dinitrophenylhydrazine in 2N HCl. The precipitate was filtered off, washed with water, dried and the OCH₃ content was determined. From the other part of the aldehyde fraction the aldehydes were extracted with ether, this was removed and the aldehydes sublimed in a high vacuum.

A particularly remarkable fact is the total absence from the plant lignins investigated by us of syringaldehyde, a characteristic component of the aldehyde fraction of hard wood lignins. When the aldehydes were distilled at a pressure of 0.1 mm no fraction could be obtained with a higher b.p. than 150° (syringaldehyde distils at 220–250° at this pressure). In the distillate, vanillin and *p* hydroxybenzaldehyde could be separated, according to Creighton & Hibbert (1944), by means of their different solubilities in benzene

and identified by mixed melting points. The proportion of the two aldehydes could be calculated in each case from the OCH₃ content of the 2,4-dinitrophenylhydrazones.

The total yields of aldehydes and the proportions of vanillin and *p* hydroxybenzaldehyde are given in Table 5. It may be mentioned for comparison that Creighton *et al.* (1944) isolated 35–51% of aldehydes (syringaldehyde and vanillin) from hardwood lignins and 15–24% of vanillin from softwood lignins. *p* Hydroxybenzaldehyde is not present in the aldehyde fraction of wood lignins and only 1.8% was obtained by Creighton & Hibbert (1944) from maize cob lignin.

Table 5 shows that the quantity of aldehydes obtained from the oxidation of plant lignins with nitrobenzene varies within wide limits, while the aldehyde fraction is almost completely absent from the nitrobenzene oxidation product of faecal lignins. It is remarkable that Gramineae lignins give a far larger quantity of aldehydes on oxidation than lignins from Leguminosae. Here, too, the botanical family to which the plant belongs has a far greater influence on the composition of the lignin than the degree of 'woodiness', as may be seen in the case of peanut hay, which belongs to the Leguminosae. Though the total percentage of aldehyde obtained varies greatly, the ratio of vanillin to *p* hydroxybenzaldehyde remains constant throughout and is approximately 2:1, without being influenced either by the botanical family or the degree of woodiness of the plant from which the lignin is derived.

The absence of the aldehyde fraction from the oxidation products of faecal lignins shows that in the body the lignin undergoes a change, which renders it incapable of yielding aldehydes on degradation with nitrobenzene.

Table 5 *Formation of aldehydes by oxidation of lignins and nitrobenzene*

Lignin from		Aldehydes (as percentage of the lignin)	Vanillin (as percentage of total aldehydes)	<i>p</i> Hydroxy benzaldehyde (as percentage of total aldehydes)
<i>Eragrostis tef</i>	Plant	19.2	64.3	35.7
	Faeces	0	0	0
<i>Pennisetum</i>	Plant	16.3	63.8	36.2
	Faeces	0.31	0	—
<i>Setaria italica</i>	Plant	16.1	66.1	33.9
	Faeces	0.26	—	—
Wild barley	Plant	21.6	66.6	33.4
	Faeces	0.25	—	—
Berseem clover	Plant	4.2	65.4	34.6
	Faeces	—	—	—
Fahli clover	Plant	4.6	66.0	34.0
	Faeces	0.15	—	—
Peanut hay	Plant	8.3	64.7	35.3
	Faeces	0.10	—	—
Cyprus vetch	Plant	6.3	62.6	37.4
	Faeces	0.04	—	—

Benzoic acid and phenol were the main constituents of the acid and phenolic fractions, respectively, no importance can, however, be attributed to them, since they probably originated from the nitrobenzene, as was shown by oxidizing sugars instead of lignins by the same method, when no aldehydes, but considerable quantities of both benzoic acid and phenol were formed

(2) *Degradation of the lignins by alkali fusion* The lignins were fused with KOH at 250–300° according to the method of Heuser & Winsvold (1923). A total of 100 g of each lignin was treated in batches of 5 g. The melt was diluted with water, acidified and extracted with ether. After evaporation of the ether, catechol and protocatechuic acid could be separated in the residue by their different solubilities in benzene and identified by colour reactions (with FeCl_3 and AgNO_3), and by identification of their methylation products, veratrol and veratric acid (CH_2O contents and mixed melting points)

Lignins from Gramineae yield 1.36–2.25 % of protocatechuic acid and 0.20–0.40 % of catechol on alkali fusion. Lignins from Leguminosae yield 0.20–1.00 % protocatechuic acid and 0.30–0.56 % of catechol. In this case, no difference between plant and faecal lignins could be found.

(3) *Treatment with acid ethanol (ethanolysis)* By 'ethanolysis', i.e. by refluxing the lignins for 40–100 hr with absolute ethanol containing 3 % by weight of HCl, Cramer, Hunter & Hibbert (1939) obtained a considerable number of well defined degradation products of wood lignin. We attempted to use this method for the investigation of plant lignins, but obtained only small amounts of degradation products, most of the lignin being converted into a black carbonized mass. For this reason we modified this method by using aqueous ethanol instead of absolute, and by working under pressure, which permitted work at a higher temperature and, therefore, a very considerable reduction of the reaction time.

The detailed procedure was as follows. 10 g of lignin were dissolved in 40 ml of 2.5N NaOH with gentle heating, and this solution was mixed with 350 ml of 85 % (v/v) ethanol

to which 10 ml of conc H_2SO_4 had been added. Ten batches of each lignin were treated in this manner. The solution of the lignin in NaOH and its reprecipitation with acid yielded a finely divided, reactive product. This mixture was now put in an autoclave with glass lining, and in the course of 2 hr heated to 180–190°, at which temperature it was kept for 6–7 hr. The black solid residue was filtered off, dried, extracted with ether, and again subjected to the same treatment in 10 g batches with acidified ethanol. After the ethanolysis had been repeated three times, there were usually only small quantities of unreacted lignin left. All solutions were now combined and 0.25 vol of water was added. After evaporation of the ethanol *in vacuo*, the residue was extracted with ether, and the ether extracted successively with NaHSO_3 , NaHCO_3 and NaOH solutions, and thus divided into aldehyde, phenol, acid and neutral fractions.

The yields of the various fractions are given in Table 6. As in the case of nitrobenzene oxidation, treatment by acidified ethanol yields much larger quantities of carbonyl compounds from Gramineae than from Leguminosae lignins. In both cases, practically no carbonyl compounds were formed from faecal lignins. The composition of the aldehyde fraction obtained by ethanolysis is, however, completely different from that obtained by nitrobenzene oxidation. The main part of this fraction was a light yellow oil which, after distillation under 0.05 mm, partially crystallized on cooling. It was assumed that this was a benzene derivative containing a side chain with an aldehyde or ketone group in it, many such compounds have been found by Hibbert (1942) and Freudenberg (1939). By addition of a Ni salt and hydroxylamine a brown complex Ni salt could be prepared, whose Ni content (determined by dimethylglyoxime after treatment with H_2SO_4) agreed with that calculated for 1-(4'-hydroxy-3'-methoxyphenyl)propane-1,2-dione dioxide (11.63 % Ni). This Ni salt was prepared according to Kulka, Hawkins & Hibbert (1941). 1-(4-Hydroxy-3'-methoxyphenyl)propane-1,2-dione is the only material obtained in this investigation with a side chain containing three carbon atoms.

The phenol fraction was separated by fractional distillation into two portions, b.p. 180–190° and 200–210°. After redistillation of each fraction, phenol and guaiacol were identified as their principal components by colour reactions (with FeCl_3 , and with FeCl_3 and AgNO_3 , respectively) and by the mixed melting point of their 3,5-dinitrobenzoates.

Table 6 Amounts of degradation products obtained by ethanolysis of lignins

(As percentage of the starting material)

Lignin from	Aldehydes	Phenol	Acids and neutral fraction	Total yield
<i>Eragrostis tef</i> Plant	2.31	6.40	4.28	12.99
Faeces	0.22	5.77	5.22	11.20
<i>Setaria italica</i> Plant	1.98	6.24	2.32	10.54
Faeces	0.22	4.00	3.45	7.67
Wild barley Plant	2.56	7.83	3.22	13.61
Faeces	0.18	2.20	3.18	5.56
Bersem clover Plant	0.61	1.28	0.42	2.31
Faeces	—	0.96	1.75	2.71
Fahli clover Plant	0.84	2.65	0.33	3.82
Faeces	0.04	1.21	2.02	3.27
Peanut hay Plant	0.98	4.50	2.07	6.55
Faeces	0.26	1.80	3.74	5.80

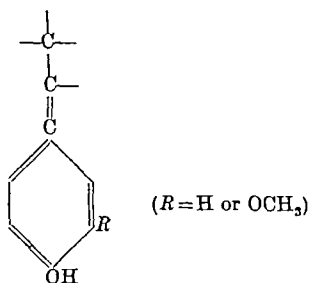
Vanillic acid was found along with *p* hydroxybenzoic acid, in the combined acid and 'neutral' fractions (the latter being the compounds remaining after aldehydes, acids and phenols were removed) The 'neutral' and acid fractions were worked up together, because the acid fraction was always obtained in very small quantities only The two fractions were subjected to oxidation with KMnO_4 by the following method

The residue remaining after evaporation of the ether was dissolved in a little ethanol, 50 ml of water were added and the mixture heated on the water bath An excess of 4% KMnO_4 solution was then added The mixture was heated on the steam bath for 1 hr and filtered The filtrate was acidified with H_2SO_4 and extracted with ether The ether was evaporated and the residue distilled under 0.05 mm Two fractions were obtained One had a very low OCH_3 content (0.2%), and could be purified by digestion with ice cold benzene and a second sublimation under high vacuum Its identity with *p* hydroxybenzoic acid was proved by the m.p. and mixed m.p., 213–215°, with an authentic specimen, m.p. 215° The fraction obtained at higher temperature could be recrystallized from water and was afterwards resublimed in a high vacuum Its m.p., 205–206°, and OCH_3 content proved its identity as vanillic acid

DISCUSSION

The structure of the lignins

The degradation products obtained by us correspond to those obtained from wood lignins It seems, therefore, that the unit



assumed by Klason (1936) and by Hibbert (1942) and Freudenberg (1939) to be present in wood lignins is also an essential constituent of the lignins in young plants (see also Percival, 1942) The occurrence of vanillin and *p* hydroxybenzaldehyde can easily be explained by the oxidative fission of the side chain double bond From model experiments carried out recently by Wacek & Kratzl (1947) it is apparent that only those aromatic compounds which contain an olefinic double bond between carbon atoms 1 and 2 in the side chain, e.g. 4 hydroxy 3 methoxycinnamic acid, give, on oxidation with nitrobenzene, vanillin in good yield, while compounds like 3,4 dimethoxybenzylacetone do not The assumption that the aldehydes formed by nitrobenzene oxida-

tion are derived from compounds containing a three carbon side chain is supported by the finding of 1 (4' hydroxy 3' methoxyphenyl)propane 1,2 dione However, the side chain is not split by ethanolsis and, therefore, no aldehydes can be found Vanillic, *p* hydroxybenzoic and protocatechuic acids result from a complete oxidation of the side chain to COOH , which is followed in some cases by the loss of CO_2 , thus leading to the formation of catechol, guaiacol and phenol The constant proportion of vanillin to *p* hydroxybenzaldehyde (2:1) observed by us seems to point to the fact that a lignin structural unit contains three aromatic nuclei, two yielding vanillin on oxidation and one *p* hydroxybenzaldehyde (the loss of a OCH_3 group by oxidation with nitrobenzene has never been observed and seems improbable)

As already pointed out on p. 253, wood lignins yield, on oxidation with nitrobenzene, considerable quantities of aldehydes rich in OCH_3 , a fact which is in accordance with the larger OCH_3 contents of wood lignins in general

The OCH_3 content of the Gramineae lignins shows that each structural unit (assuming its molecular weight to be 625) must contain two OCH_3 groups (theory, 9.9% OCH_3), see Table 3 Methylation with diazomethane raises the OCH_3 content to 19%, which shows that two phenolic OH groups are methylated Since the experimental evidence points to the presence of three aromatic rings, two of them substituted with OCH_3 and OH and the third with OH only, one must necessarily assume that the remaining phenolic OH group is attached in such a position as to make it incapable of being methylated with diazomethane

Leguminosae lignins contain only one OCH_3 group per structural unit (theory, 5.1%) and show on methylation with diazomethane an increase in OCH_3 content which corresponds to the presence of two phenolic OH groups Since, on oxidation with nitrobenzene of the Leguminosae lignins, vanillin and *p* hydroxybenzaldehyde are also formed in the proportion 2:1 (although in smaller yields), one must assume that part of the units still contains a system of two rings, each of them substituted with OH and OCH_3 , and one ring substituted with OH only This would mean that half of the Leguminosae lignin is methylated in the usual manner, i.e. it contains two OCH_3 groups in one structural unit The other half is not methylated at all A large part of the free phenolic OH groups in Leguminosae lignins is apparently incapable of being methylated.

Plant lignins take up one OCH_3 group more on methylation with dimethyl sulphate than on methylation with diazomethane indicating the presence of one non phenolic OH group

Plant lignins contain, in contrast to wood lignins, nitrogen as a characteristic component All lignins

can be classified by their increasing N and decreasing OCH_3 content as follows

- Hardwood lignins, N, nil, OCH_3 , 20 %
- Softwood lignins, N, 0.2–0.3 %, OCH_3 , 15 %
- Gramineae lignins, N, 1.2–1.6 %, OCH_3 , 10 %
- Leguminosae lignins, N, 2.9–3.4 %, OCH_3 , 5 %

Digestion of lignins

The solubility of the lignins, the apparent molecular weight of the building units, and the OCH_3 and N content remain unchanged by digestion. On the other hand, the disappearance of the aldehyde fraction from among the degradation products of faecal lignins shows that changes in the side chains of the aromatic rings have occurred, probably by hydrogenation of the side chain double bond, preventing their oxidative fission and the formation of aldehyde groups. The other significant change is the complete disappearance of the non phenolic OH group indicated by the fact that the increase in OCH_3 content, obtained by methylation with dimethyl sulphate, is identical with that obtained with diazomethane.

SUMMARY

1 Lignins were prepared by extraction with dilute alkali from a number of annual forage plants and from faeces collected from sheep fed exclusively with the same plants. The plants used were *Eragrostis tef*, *Pennisetum*, *Setaria italica*, *Hordeum murinum* (wild barley), *Trifolium alexandrinum* bersem, *T. alexandrinum fahli*, *Lathyrus ochrus* and hay of *Arachis hypogaea* (peanut).

2 All lignins contained nitrogen whose presence is not due to accidental contamination with proteins, but seems to be a characteristic component of the lignin molecule itself. The methoxyl content of the lignins ranges from about 5 % in Leguminosae lignins to about 10 % in Gramineae lignins.

3 On methylation with diazomethane, the original methoxyl content of the Leguminosae lignins is trebled, that of the Gramineae lignins doubled. Methylation with dimethyl sulphate brings about a small increase in methoxyl uptake over that obtained with diazomethane. In faecal lignins no additional methylation with dimethyl sulphate could be obtained.

4 Cryoscopic molecular weight determinations in β naphthol showed values of about 600 both for plant and faecal lignins.

5 Oxidation of the plant lignins with nitrobenzene in alkali yielded vanillin and *p*-hydroxybenzaldehyde. Faecal lignins did not yield any aldehydes. The ratio of vanillin to *p*-hydroxybenzaldehyde was always 2:1.

6 On fusion with potassium hydroxide all lignins gave catechol and protocatechuic acid.

7 On heating the lignins with ethanol containing 3 % sulphuric acid to 170–180° under pressure, 1-(4'-hydroxy-3'-methoxyphenyl)propane 1,2-dione was obtained only from plant lignins, both plant and faecal lignins yielded phenol, guaiacol and an acid fraction, which, after further oxidation with KMnO_4 , yielded vanillic and *p*-hydroxybenzoic acids.

8 The experimental results are discussed in relation to the structure of the lignins.

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Penicillin Analysis of the Crude Product by Means of a Modified Partition Chromatogram

1 THEORY-AND USE OF THE CHROMATOGRAM

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Crude penicillin isolated from solvent extracts of metabolism solutions (Abraham, Chaim, Fletcher, Florey, Gardner, Heatley & Jennings, 1941, Abraham, Chaim & Holdday, 1942), as a calcium, barium or sodium salt, is a yellow to brown solid which may contain well over thirty different acidic substances, mostly coloured. Still further weakly acidic and neutral substances remain in the residual solvent phase. The physical and chemical properties of the substances are such that chromatography provides the only likely method for the separation of the components of the mixture. So far, however, few adsorbents have been found which give satisfactory chromatograms. A modified partition chromatogram has been described (Catch, Cook & Heilbron, 1942) based on that of Martin & Synge (1941), but this chromatogram is capable of little development although considerable purification can be achieved.

The present paper describes work begun in 1942 on a modification of the Martin & Synge (1941) partition chromatogram, which has been found suitable for substances of the nature of the acids in crude penicillin, and which can be developed. This chromatogram consists of silica gel impregnated with strong solutions of potassium phosphate of a suitable acidity. With this technique it has been possible to demonstrate in crude penicillin the existence of at least five chemical individuals with varying degrees of antibacterial power, although complete purification of any one of them by this method alone has not been achieved.

In the earlier stages of this work the position of the active zones among the numerous visible coloured bands could only be located by biological activity, the test results for which were not available until 24 hr after the column had been run. Partition coefficient determinations in tap funnels under conditions comparable to those on the columns were, therefore, carried out and the results applied to the columns according to the theoretical treatment of Martin & Synge (1941). This proved a valuable guide to the approximate position of the main active zone, but as larger columns and heavier loads were used it became apparent that this treatment was inadequate,

as indeed its authors imply (Martin & Synge, 1941). By this time experience enabled us to locate the desired bands with some certainty from the appearance of the column, and from the position of a prominent orange band associated with the colour reaction described by Catch *et al* (1942). On our column this band was substantially inactive and occurred a little below the main active zone. Still later the polarimeter was of great assistance because the biological activity was always associated with a strong dextrorotation, other constituents being usually laevorotatory or optically inactive.

It was clearly desirable to define as closely as possible the best conditions for carrying out various types of separations, since it was obvious that there was much overlapping of bands. In particular the separations of penicillins I, II and IV proved to be very difficult (cf Part 2, Boon, Calam, Carrington & Freeman, 1944, Boon, Carrington & Levi, 1944). An attempt was made, therefore, to apply the recent theories of de Vault (1943) and Weiss (1943) to this type of column. A practical test of the theory was then made with phenylacetic acid and benzoic acid as model substances.

THEORETICAL TREATMENT

Consider a column prepared as described below of length L cm, and cross sectional area A sq cm containing M g of silica gel (sp gr D) impregnated with K ml of a buffer solution. The column is initially filled with solvent.

Area of silica phase = $M/LD = I$

Area of buffer phase numerically equal to volume of buffer/cm of column = $K/L = P$

Area of solvent phase numerically equal to pore volume/cm of column = $A - I - P = S$ (Martin & Synge, 1941)

Consider a single solute added to the column in a volume of solvent v_0 , so that its concentration is C_0 .

Let V be the total volume of filtrate at any time, and v the volume of filtrate after the commencement of development where $v = V - v_0$.

Let x be the distance of any point from the top of the column.

Let C be the concentration of the solute at any point in the solvent phase, and $B (=f(C))$ be the concentration in the buffer phase.

For the partition chromatogram the differential equation deduced by de Vault (1943) becomes

$$\frac{\partial C}{\partial x} + \frac{\partial C}{\partial v} [S + Pf'(C)] = 0, \quad \text{where } f'(C) = \frac{dB}{dC} \quad (1)$$

$$\text{or} \quad x = F(C) + \frac{v}{S + Pf'(C)}, \quad (2)$$

where $F(C)$ is an arbitrary function to be defined by the initial conditions

From this equation and a detailed consideration of the processes on the column analogous to that of de Vault (1943) and Weiss (1943) we obtain the following relations

Initial width of band (x_1) This can be found from equation (2), or more simply as follows

Of the total quantity of solute $v_0 C_0$ the amount in the mobile phase is $x_1 S C_0$, and the amount in the stationary buffer phase is $x_1 P f'(C_0)$

$$\text{Hence} \quad v_0 C_0 = x_1 S C_0 + x_1 P f'(C_0)$$

$$\text{or} \quad x_1 = \frac{v_0 C_0}{S C_0 + P f'(C_0)} = \frac{v_0}{S + P (B_0/C_0)} \quad (3)$$

Lower edge of constant concentration zone (x_2) Since the concentration in the solvent phase at the front of the band is still C_0 the lower edge of the zone moves as though the original solution were still being poured into the column. Hence we can write, as for x_1 ,

$$x_2 = \frac{V C_0}{S C_0 + P f'(C_0)} = \frac{V}{S + P (B_0/C_0)} \quad (4)$$

Upper edge of constant concentration zone (x_3) For the point x_2 if volumes are measured from the commencement of development $F(C) = 0$ (de Vault, 1943) and equation (2) becomes

$$x_2 = \frac{v}{S + P f'(C_0)} \quad (5)$$

Concentration in the 'tail' Concentrations can only be conveniently measured as the band leaves the column, i.e. when $x = L$. Measuring v from the commencement of development as before $F(C) = 0$, and

$$L = \frac{v}{S + P f'(C)} \quad \text{or} \quad f'(C) = \frac{v}{LP} - \frac{S}{P} \quad (6)$$

From this expression concentrations at any point in the 'tail', as it emerges from the column can be determined.

Volume (v_m) at which the constant concentration zone just disappears When this occurs $x_2 = x_3$. On equating the expressions for these we obtain

$$v_m = \frac{v_0 C_0 \{S + P f'(C_0)\}}{P \{f'(C_0) - C_0 f''(C_0)\}} \quad (7)$$

It is seen that the theory of Martin & Synge gives an equation of the same form as (3) and (4) above, the only difference being the substitution of partition ratios for partition coefficients

EXPERIMENTAL

Distribution experiments with benzoic and phenylacetic acids

Partition coefficients of phenylacetic acid at different acidities using concentrated buffer solutions A stock solution of phenylacetic acid (2.72 g, 20 m equiv) in damp diethyl

ether (100 ml) was prepared. Of the solution 10 ml required 18.5 ml of approximately 0.1N NaOH. A series of buffer solutions covering the approximate range pH 4.5–7.5 was prepared by mixing suitable volumes of 25% (w/w) KOH and H_2PO_4 until the desired pH was obtained after dilution 1 in 4. The solutions were saturated with ether before use. Quantities of the stock solution (2 ml) were diluted to 20 ml with ether, and each was shaken with 20 ml of one of the buffer solutions. The pH of the buffer phase was measured after the partition. Sample parts of the ether phase were titrated. The concentration of acid in the buffer phase was estimated by difference. The results are shown in Fig. 1.

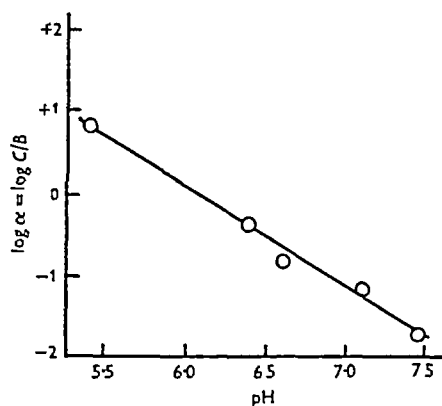


Fig. 1 Relation between partition ratio and pH for phenylacetic acid in ether and strong buffer solutions

Effect of concentration on distribution between the phases

(a) *Phenylacetic acid* The procedure was similar to that used in the previous experiment, with one buffer solution (pH 6.84) and solutions of phenylacetic acid in diethyl ether of varying concentration. Concentrations are conveniently expressed in m equiv/l. The results are given in Table 1.

(b) *Benzoic acid* The experiment was carried out in the same way as the one for phenylacetic acid using buffer solutions of pH 6.72. The results are given in Table 2.

Partition chromatogram with phenylacetic acid

Silica gel (Martin & Synge, 1941, Gordon, Martin & Synge, 1943) (90 g, 90–160 mesh) was thoroughly mixed in a mortar with concentrated potassium phosphate buffer prepared as previously described (pH 6.36, 45 ml) and the resulting, apparently dry, powder was suspended in ethylene dichloride (500 ml.) and poured into a chromatogram tube (3.68 cm diam). After the column had ceased to shrink (final dimensions 27 × 3.68 cm) the ethylene dichloride was displaced with diethyl ether. Phenylacetic acid (2 g) in diethyl ether (200 ml) was then applied to the top of the column, and development was effected with moist ether, 200 ml of filtrate were collected as a first fraction followed by further fractions of 20 ml. The concentration of phenylacetic acid in the eluate was determined by titration with standard $Ba(OH)_2$. For this column the following values were calculated by the method of Martin & Synge (1941)

Pore vol./cm numerically equal to area of solvent phase (S) = 7.49 ml

Vol of buffer/cm numerically equal to area of buffer phase (P) = 1.67 ml

Table 1 *Effect of concentration on the distribution of phenylacetic acid between ether and concentrated buffer solutions*

(Acidity of buffer solutions was pH 6.82)

Concentration in ether phase (m equiv./l.)	log	Buffer phase (m equiv./l. observed)	log	Buffer phase calculated from regression equation (m-equiv./l.)
2.66	0.4249	14.3	1.1553	15.2
5.8	0.7634	30.8	1.4886	27.6
15.2	1.1818	56.1	1.7490	57.4
15.3	1.1847	57.8	1.7619	57.7
19.7	1.2945	69.6	1.8426	70.0
44	1.6435	134	2.1271	128
104	2.0170	242	2.3838	245

Table 2 *Effect of concentration on the partition of benzoic acid between ether and concentrated buffer*

(Acidity of buffer solutions was pH 6.72)

Concentration in ether phase (m equiv./l.)	log C	Concentration in buffer phase (m equiv./l.)	log B
27.6	1.44	69.3	1.84
59.8	1.78	123	2.09
168	2.23	239	2.38

Chromatograms with mixtures of benzoic and phenylacetic acids

Results of four experiments are illustrated in Fig. 2. The experiment illustrated in Fig. 2A was carried out with the same column as used for the above experiment with phenyl

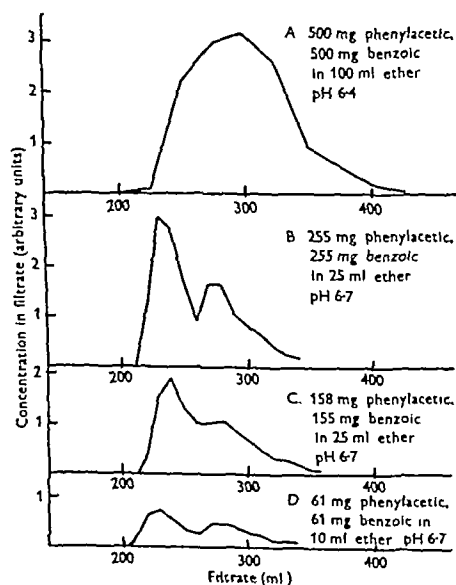


Fig. 2 Mixtures of phenylacetic and benzoic acids, partition chromatogram

acetic acid. The other three were carried out with a single new column differing only slightly in dimensions but with a buffer solution of higher pH (6.7)

DISCUSSION

In order to evaluate the expressions obtained in the theoretical portion it was first necessary to evaluate $f(C)$, i.e. to determine the effect of concentration on the partition ratio. For benzoic and phenylacetic acids the experimental results can be satisfactorily expressed in the form $f(C) = B = aC^b$ resembling the Freundlich adsorption isotherm where a and b are constants for the particular substance and pair of phases. It probably has little theoretical significance, and only holds for a comparatively narrow range of conditions since at low concentrations the value of B/C should approach a constant value whereas the above expression would make B/C continue to increase with decreasing C , i.e. b is not truly constant, but tends to unity as the concentration decreases. Nevertheless, over the range of concentrations normally employed in chromatography, the expression is sufficiently accurate for most purposes.

The calculated regression line for the results given in Table 1 is $\log B = 0.86 + 0.76 \log C$, i.e. $B = 7.2 C^{0.76}$ at pH 6.84. At pH 7.4, $a = 16.98$, $b = 0.76$. The corresponding figures for benzoic acid at pH 6.72 are $a = 7.24$, $b = 0.68$. The value of b will probably normally lie well within the limits 0.5 and 1.0 (Note 1). The value of a is the value of B when $C = 1$, and approximates to the limiting partition ratio if suitable units are chosen.

The best fitting equation for the line shown in Fig. 1 is $\log (C/B) = 7.32 - 1.20 \text{pH}$. By means of this result and the value $b = 0.76$ obtained above for phenylacetic acid it is possible to calculate $f(C)$ and $f'(C)$ for the chromatogram using phenylacetic acid. The appropriate expressions are $f(C) = 2.82 C^{0.76}$,

$f'(C) = 2.14 C^{-0.21}$, and from these the following may be calculated

Position of front edge of band 250 ml (from equation (4)),

End of constant concentration band 436 ml (from equation (5))

These are in reasonably good agreement with the experimental results, particularly at the front edge of the band, as the small overlap shown in Fig 3

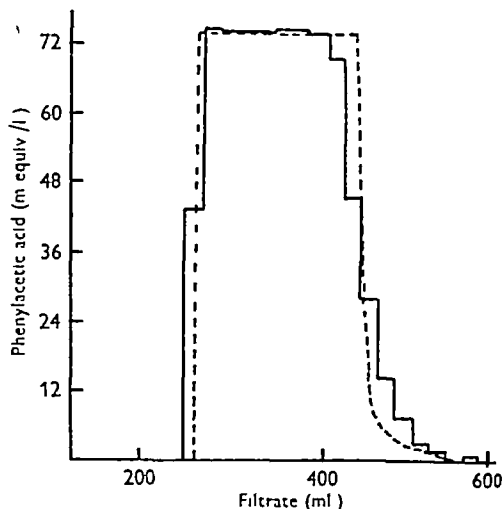


Fig 3 Phenylacetic acid on a partition chromatogram
Full line, observed, dotted line, calculated

is due to the fact that fractions were taken at 240 and 260 ml, and thus would include some eluate containing no acid. This sharpness of the front edge of the band indicates that other factors (diffusion, non-attainment of equilibrium, etc.) are relatively unimportant. The observed tail end of the constant concentration zone appeared a little earlier, and the fall off in the tail was slower than anticipated. It is thought that this is almost entirely due to the practical impossibility of achieving sharp boundary conditions when changing from solution to pure solvent. Thus, even if there is little mixing of the layers of liquid where they enter the column, some solute will be washed down from the walls of the tube above the column, especially with volatile solvents. In earlier experiments with this technique it was usual to add washings from the various vessels to the column, after the bulk of the charge had been added, for the sake of a good recovery. This was clearly a mistake and made the banding unnecessarily diffuse.

The results for the chromatograms of mixtures of benzoic and phenylacetic acids are of particular interest since the difficulty in separating acids was of about the same degree as the separation of penicillins I and II. In experiment A not only is

the pH low, but the initial volume is too great so that the bands largely overlap. The higher pH and lower concentrations of experiments B, C and D proved much more effective (Fig 2). These experiments were not carried further as ample examples of the practical application of the technique were obtained during the course of its use with crude penicillin (see Boon, Calam, Gudgeon & Levi, 1948).

It is probable that it will seldom be of value to apply the above equations in practice (especially since no account has been taken of the effect of one band on another) except in the case of colourless substances, or for the purposes of identification, and indeed our main interest in the theory was to define the best conditions for the treatment of the very complex mixture in crude penicillin. In this case, before the isolation of the pure substances, $f(C)$ cannot be determined so that a rigid application of the theory is impossible. Thus, as will be shown in later papers, many of the constituents occur close together on the column, and the theory was of assistance in defining conditions under which they could be separated.

Some of the practical consequences of the theory are set out below.

(1) On a given column the initial width of a band depends mainly on the total volume of the starting solution (equation (3)) relative to the size of the column. If the equation is written in the form

$$x_1 = \frac{v_0}{S + (PB_0/C_0)} = \frac{v_0}{S + PaC_0^{b-1}},$$

and taking the values quoted above for b , it is seen that for a given v_0 the band width will vary only slowly with change of concentration. The band width will be less at higher pH values (increasing a).

(2) As a corollary to this, to separate two substances whose partition relations lie close together on a given column, a small initial volume and a high pH will give the best conditions. It will be preferable that the constituent in greater amount should be the less soluble in the buffer phase, since the rate of movement of the band is increased by increased concentration.

(3) It is desirable that the material in a band should be mainly concentrated at the head of the band, and not too spread out into the 'tail', i.e. v_m in equation (7) should be a maximum. (It is not, however, essential that v_m should not be exceeded.) Substituting the values for $f(C)$ and $f'(C)$ in equation (7) we have

$$v_m = \frac{v_0 C_0 S}{PB_0(1-b)} + \frac{v_0 b}{(1-b)}$$

This expression is a maximum when v_0 is large, and when the pH is low. These conditions are the opposite of those in (2) above, so that in practice compromise conditions must be sought. Prolonged

development will give a wider separation at the front edges of the bands, but owing to the increased diffuseness the total separation may not be greatly increased in some cases. In any case it is clear that development should not be unduly prolonged.

An interesting feature of the above expression is that it is independent of the diameter of the column. This gives a definite advantage to a short wide column over a long narrow one owing to its greater practical convenience in working. This must not be overdone, however, as too rapid a solvent flow gives diffuse columns, due mainly to the fact that attainment of equilibrium is not instantaneous.

(4) Provided suitable conditions of quantity, concentration, partition ratio and degree of development are chosen, a single solute should give a band after development on a column with

- (a) a sharp front edge, followed by
- (b) a constant concentration zone, and
- (c) the concentration in the solvent phase in equilibrium with the constant concentration zone should be equal to the concentration of the initial solution

If a substance is found to give, for example, a band with a maximum near its top edge then it is not homogeneous. It is assumed, of course, that the banding on the column is regular.

Practical illustrations of the above considerations will be found in the subsequent papers of this series. They will apply *mutatis mutandis*, to all types of chromatogram except that, in the case of partition

chromatograms of this type, the effect of one band on another will always be considerable because solution of the acids in the stationary phase will lower the pH. On the other hand, in many cases the direct adsorption of one substance by, for example, alumina is apparently not greatly affected by the adsorption of another.

Partition chromatograms of this type are clearly not limited to acids. With suitable buffers, bases should be separable (cf Catch *et al* 1942). It might also be possible, for example, to separate mixtures of aldehydes and ketones, using columns impregnated with bisulphite solution. The necessary conditions for a separation are clearly an adsorbed stationary phase and a moving phase, between which some or all of the substances to be separated can be differentially and reversibly distributed.

SUMMARY

1 A modified partition chromatogram for the separation of acids is described. It is based on that of Martin & Synge (1941), but concentrated potassium phosphate buffer solutions replace water as the stationary phase.

2 The theories of de Vault (1943) and Weiss (1943) are shown to give an adequate account of the behaviour of a single solute on such a chromatogram.

3 The effect of concentration on the distribution of phenylacetic acid or benzoic acid between diethyl ether and concentrated buffer solutions is expressible by an equation of the same form as the Freundlich adsorption isotherm.

Addenda (1947)

(1) The limits suggested for likely values of b in the equation $B = aC^b$ are too narrow. Acids have been encountered for which values of $b < 0.5$ have been found.

(2) The work originally intended to be published as part 3 in this series will be published in a forthcoming monograph on penicillin chemistry.

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Penicillin Analysis of the Crude Product by Partition Chromatography

2 CHROMATOGRAPHIC ANALYSIS OF THE PENICILLINS FROM TWO STRAINS OF *PENICILLIUM NOTATUM*

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This paper is the second of a series dealing with the analysis of penicillin by means of a chromatogram using solvent phosphate buffer partition on a silica base. The first paper (Levi, 1948) described the development of this type of chromatogram which is based on that of Martin & Syngé (1941), and outlined the principles of its application to the study of penicillin.

The work described was carried out mainly in 1943, and communicated privately to collaborating laboratories in Great Britain and the United States at the end of July in that year. Wider publication was not possible at that time owing to wartime secrecy restrictions. It deals with an examination of the crude penicillin produced by two strains of *Penicillium notatum* grown under different conditions, and shows clearly that the main active constituents of the two products are different, a fact which was not appreciated until the work was carried out. The further examination of the crude product is also described and shows the existence of still other penicillins.

EXPERIMENTAL

Materials. Two types of crude penicillin were used. The first was prepared by growing a strain of *P. notatum* (Imperial Chemical Industries Ltd, ref. F4) obtained from Prof. H. W. Florey in surface culture on a Czapek Dox medium with the addition of 0.5% acid hydrolyzed casein. From the resulting broth the penicillin was isolated in the usual manner, by solvent extraction, as a calcium salt of activity 100–150 units/mg.

A sample of the second type was generously supplied to us by Merck and Co. Inc., Rahway, U.S.A. It was prepared by growing the pre-Fleming strain N.R.R.L. no. 832 (supplied by the Northern Regional Research Laboratory, U.S. Department of Agriculture) in submerged culture in a brown sugar corn steep liquor medium from which it was isolated as a sodium salt.

Biological assay. Two methods of assay were employed: a serial dilution method with *Staphylococcus aureus*, and a cylinder plate method with *Bacillus subtilis* as the test organisms. In both cases a sample of calcium penicillin prepared from F4 strain penicillin was used as standard. In the case of the F4 material the activity determined by

either method was the same, but with the 832 strain material the activity, as measured by the *B. subtilis* method, was significantly higher than when measured against *S. aureus*.

Treatment of crude penicillin with alumina. A preliminary treatment of the crude penicillin by this method greatly facilitated the subsequent chromatographic analysis by removal of much inert material. In a series of experiments it was found to raise the activity of the F4 strain material from 60–120 units/mg to 260–330 units/mg, and in the case of the 832 strain material from 165 to 474 units/mg.

A typical experiment was as follows: crude calcium penicillin (22 g) was dissolved in water (1.8 l) and the solution was cooled in ice and stirred. Activated alumina (Type 'O', as supplied by Peter Spence and Sons Ltd, Widnes, 330 g) was gradually added during 10 min, and stirring was continued for 1 hr after which the alumina was filtered off and washed twice by stirring for 5 min with 250 ml water. The combined solution and washings were mixed with chloroform (2 l), cooled to 3°, and the penicillin extracted into chloroform by acidifying to pH 2 with 4M H_3PO_4 (60 ml). The aqueous layer was quickly re-extracted with cold chloroform (1 l). The extract after washing with water, was stirred successively with three lots of water (300, 200, 100 ml) containing precipitated chalk (30, 20 and 10 g respectively). After filtration and separation the solution of calcium penicillin was dried from the frozen state. Weight of product, 6.47 g, 314 units/mg (yield 81%).

Chromatographic analysis of penicillin preparations

Preliminary silica phosphate buffer chromatogram of F4 strain penicillin. Silica (60–160 mesh, 185 g) was mixed with 25% (w/w) potassium phosphate buffer (pH 6.27, 90 ml) and diethyl ether (10 ml) in a mortar. The powder was suspended in ethylene dichloride (1 l) and stirred for 1 hr, the resulting slurry was then poured into a long chromatogram tube containing a few cm of ethylene dichloride. Solvent was allowed to run through the column until it ceased to shrink, the next day ether was run through until there was again no further shrinkage. The final dimensions of the column were 5.2 × 22 cm.

Alumina treated calcium penicillin (8 g, activity 298 units/mg) was dissolved in water (150 ml), and, after acidification at 3° to pH 2, extracted into ether (250, 150, 100 ml). The solution was run on the top of the column and, after it had sunk in, development was continued with ether until

most of the activity, as measured by the optical rotation in 2 dm tubes, was found in the percolate which was divided into 5 fractions. The penicillin was isolated as barium salt from these by shaking the ethereal solution with a suspension of freshly precipitated BaCO_3 , filtering, separating the aqueous layer, and drying from the frozen state. The residue on the column was recovered by suspending the silica in water (300 ml), after stirring for 5 min the suspension was filtered and the residue washed with water (200 ml). The combined filtrate and washings were cooled below 5° , acidified to pH 2 with H_3PO_4 , and the penicillin extracted with cold ether (250 and 150 ml). The penicillin was then isolated as barium salt in the same manner as the main fractions. Results are given in Table 1.

Separation of the penicillins in the mixture from F4 strain material. Early experiments suggested that the main active fraction obtained in chromatographic analysis (e.g. fractions 2-4 of Table 1) might be a doublet in which the slower moving constituent predominated. A consideration of the theory of the partition chromatogram (Lester Smith, 1942, Levi,

1948) suggested that if a larger band were the upper one it would tend to overtake a smaller one just below and thus render separation difficult. Accordingly column fractions at the head and tail of the main penicillin zone (such as fractions 3 and 5 in Table 1) were mixed and analyzed on a column 3.5×21.5 cm made as above from silica (150 g) and pH 6.5 buffer (70 ml). The penicillin (3.0 g, activity 166 units/mg) as calcium salt was transferred to ether (50 + 30 ml) and applied to the column in the usual way, development was effected with ether (930 ml). The results are given in Table 2. Fraction 2 (designated by us Penicillin V), which corresponded in properties with the first runnings from the column shown in Table 1, was shown to differ markedly from Penicillin I (fraction 5, Table 2) by a comparison of their partition coefficients between water and 25% (w/w) potassium phosphate buffer. This comparison is shown in Table 3. The bands of the active material intermediate between fractions 2 and 5 we have designated penicillin IV.

Table 1 *Fractionation of F4 strain penicillin by the partition chromatogram*

Fraction	Description of band	α_D (2 dm)	Vol of ether (ml)	Wt of salt (g)	Activity (units/mg)	Total units
1	Several pale yellow bands	-0.02°	540	0.42	12.5	5,200
2	Orange band (gives red colour with 5% H_2SO_4 in acetic acid)	$+0.05^\circ$	380	2.07	45	93,000
3	Very pale band, then yellow	$+0.05^\circ$ to $+0.3^\circ$	250	0.45	198	89,000
4	Yellow, then mostly very pale	$+0.5^\circ$ to $+0.25^\circ$	560	1.22	920	1,120,000
5	Very pale	$+0.25^\circ$ to $+0.1^\circ$	600	0.35	733	266,000
6	Residue in a column	—	—	1.07	338	362,000
	Totals —	—	—	5.85 (70%)	—	1,985,200 (81%)

Table 2 *Penicillins I, IV and V*

Fraction	Vol of eluate (ml) or length of silica (cm)	Weight (g)	Activity (units/mg)	Total units	Remarks
1	220 ml	0.12	4	480	—
2	233 ml	0.40	460	184,000	Pen V
3	268 ml	0.27	63	17,000	—
4	250 ml + 4.0 cm	0.25	419	105,000	Pen IV
5	1.7 cm	0.05	134	6,700	—
6	3.0 cm	0.11	677	74,500	Pen I
7	12.8 cm	0.68	38.5	26,200	—
	Totals	1.88	—	413,880	—

Table 3 *Partition coefficients of penicillins I and V between ether and 25% potassium phosphate buffer*

	pH	5.7	6.2	6.55	6.7
Pen V	$\alpha \frac{(\text{conc in buffer})}{(\text{conc in ether})}$	0.35	1.8	3.1	8.6
Pen I	$\alpha \frac{(\text{conc in buffer})}{(\text{conc in ether})}$	12	30	58	74

Table 4 *Preparation of penicillin III*

Fraction	Vol of eluate (ml)	Wt of calcium salt (g)	Activity (units/mg)	Total units
1	100	0.08	17	1,360
2	50	0.16	18.5	2,960
3	50	0.18	39	7,030
4	50	0.18	40	7,200
5	50	0.12	83.5	10,000
6	50	0.12	335	4,020
7	Residue in column	0.08	4	320
Totals		0.92	—	32,890

Penicillin remaining on the first column, such as fraction 6 (Table 1), was examined on a column of silica (50 g) impregnated with 25% (v/v) potassium phosphate solution of pH 5.0 (30 ml) using 1.5 g of material of activity 36 units/mg. Development was effected with ether (350 ml). Detailed results are shown in Table 4.

Partition chromatogram of 832 strain penicillin
N R R L no. 832 strain calcium penicillin (0.6 g, activity 473 units/mg) was transferred to ether in the usual way, and applied to a column (3.55 × 32 cm) prepared from silica (115 g) impregnated with pH 6.5 buffer (58 ml). After development with ether the column was divided mechanically into fractions, and the penicillin was isolated in the usual way. The results are shown in Fig. 1.

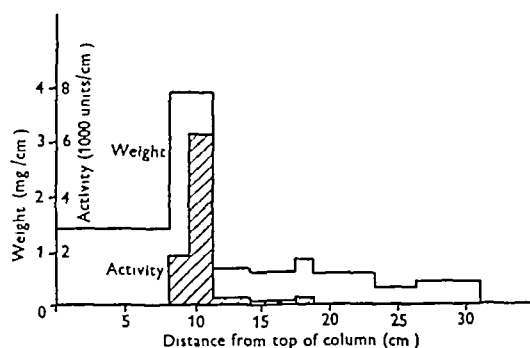


Fig. 1 Chromatogram of '832' penicillin

Separation of mixed main fractions from F4 and 832 penicillins
Sodium penicillin I fraction from F4 penicillin, similar to fraction 5 of Table 2 (123 mg, activity 750 units/mg) was mixed with the calcium salt of 832 penicillin (72 mg, activity 935 units/mg) prepared as above and transferred to ether (40 ml), and applied to a column (2.8 × 25.5 cm) prepared from silica (73 g) impregnated with pH 6.5 buffer (37 ml). Development was effected with ether (850 ml). The results are shown in detail in Fig. 2.

In order to provide a convenient scale for both column and eluate fractions the distances of movement of the bands are given in terms of their

distance from the top of the column at the end of development. For those fractions which were eluted the distance is calculated on the assumption of uniform development in a very long column. If d cm

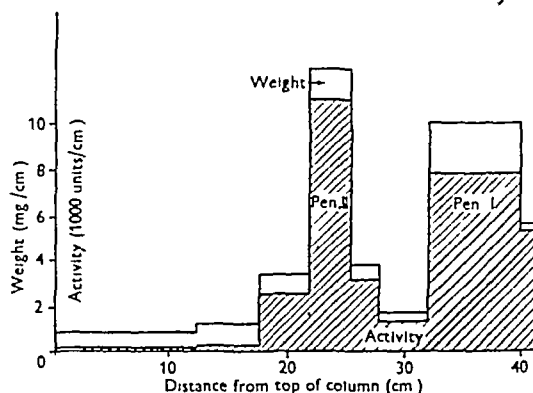


Fig. 2 Chromatogram of mixed 'F4' and '832' penicillins

is the distance that such a fraction would have moved from the top of a column of actual length l cm, and v ml is the volume of solvent required to develop it to the bottom of the column, and V ml is the total volume of solvent used, then

$$d = \frac{Vl}{v}$$

DISCUSSION

The behaviour of F4 and 832 penicillins on a phosphate buffer chromatogram differed considerably. The former material gave a series of yellow or orange bands several of which fluoresced yellow or green in ultraviolet light. The main zone of antibacterial activity was rather diffuse, and was preceded by a deep, orange, practically inactive, band associated with the colour reaction of Catch, Cook & Heilbron (1942) and a small zone of weak antibacterial activity. There was also another small antibacterial zone above the main band. Further examination of the main zone revealed that it was composed of two substances of which one was present in considerably greater quantity than the other. Thus we have

designated as penicillin I. In order to demonstrate a definite separation from the minor constituent, designated penicillin IV, it was necessary to analyze chromatographically a mixture of the head and tail fractions of the main antibacterial zone since the large quantity of penicillin I relative to penicillin IV in the zone as a whole tended to swamp the latter.

The small zone of antibacterial activity, which came out with the first runnings from the first column, has been shown to be different from penicillin I both by separation from it on a partition chromatogram and by a direct comparison of the effective distribution coefficient of the two substances in bulk. It has been tentatively designated penicillin V.

The antibacterial substance, which remained on the first column, has been shown to be different from penicillin I by the fact that it was firmly retained on a column which would not retain penicillin I at all. The exact nature of this substance has not been determined owing to the small quantity available, but it is probably the same as the penicillin III which has been recognized in N R R L no 1249 strain penicillin (Catch, Cook & Heilbron, 1944, Boon, Carrington & Levi, 1944).

The penicillin isolated by the procedure described here had a potency of 800–1250 units/mg and a purity of the order of 60%. The final purification of this material, by further chromatographic procedures followed by crystallization of the sodium salt, will be described in the forthcoming monograph on Penicillin Chemistry.

The significant difference between the biological

activity of N R R L no 832 strain penicillin against two different test organisms, when using an F4 strain standard, suggested that there was probably some difference in the constitution of the two substances. This was confirmed by chromatographic analysis. Not only was the general appearance, in visible and ultraviolet light, different from that of the F4 strain penicillin, but the main zone of activity was found to be much more compact and to develop more slowly than with the latter material. Final confirmation was obtained when a mixture of penicillin I and purified 832 penicillin was examined on a further column when a complete separation was observed. We have designated the new penicillin as penicillin II.

SUMMARY

1 Partition chromatography with a silica-phosphate buffer solvent system has shown the existence of five penicillins.

2 The main antibacterial substance produced by the F4 strain of *Penicillium notatum* grown on a modified Czapek-Dox medium is penicillin I which is accompanied by small quantities of three other penicillins.

3 The main antibacterial substance produced by the N R R L no 832 strain of *P. notatum* grown submerged in a brown sugar-corn steep liquor medium is penicillin II different from any of the above.

4 There is a differential response of two organisms, *Staphylococcus aureus* and *Bacillus subtilis*, to the action of F4 penicillin and 832 penicillin.

Addendum (1947)

At the time this work was done in 1943 the chemical difference between penicillins I and II was unknown, and we introduced the system of nomenclature by which the penicillins, as discovered, were given a roman numeral. In modern notation penicillins I, II and III are respectively

2-pentenyl, benzyl and *p* hydroxybenzyl penicillin. The exact constitution of penicillin IV has not yet been determined, but the evidence available suggests that it is an isomer of 2-pentenylpenicillin. Penicillin V may be identical with *n*-heptylpenicillin.

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Studies on the Metabolism of Semen

4 AEROBIC AND ANAEROBIC UTILIZATION OF FRUCTOSE BY SPERMATOZOA AND SEMINAL VESICLES

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Previous studies on the metabolism of semen have furnished evidence that the reducing carbohydrate in seminal plasma is fructose, which is readily converted by the spermatozoa to lactic acid, thus providing an important source of energy for the sperm cells (Mann, 1946*a, b, c*). The rate of fructolysis represents an accurate and at the same time simple means of evaluation of semen, the fructolysis index (mg fructose utilized by 10^9 spermatozoa in 1 hr at 37°) in normal bull and ram semen is 1.4–2.0, while semen with poor sperm motility gives much lower index values, azoospermic and necrospermic semen was shown to be altogether unable to metabolize fructose (Mann, 1948*a, b*). Fructose originates in the accessory glands of reproduction, mainly the seminal vesicles, but in some species it is found also in the ampullae and in certain parts of the prostate organ (Mann, 1946*c*, Davies & Mann, 1947). The process of fructose formation is initiated and controlled by the testicular hormone, a hormonal deficiency due, for example, to castration, causes invariably a decrease or disappearance of seminal fructose, but treatment with testosterone promptly restores the ability of the accessory glands to produce fructose (Mann & Parsons, 1947).

In this paper it will be demonstrated that fructolysis is a characteristic feature of both the aerobic and anaerobic metabolism of semen. Unlike spermatozoa, the seminal vesicles will be shown to lack the ability to utilize fructose anaerobically. Finally, the position will be discussed of fructolysis in relation to glycolysis and respiration, in both semen and seminal vesicles.

EXPERIMENTAL

Material. Ram semen was obtained by the method of Walton (1945), the procedure for washing the spermatozoa and the composition of the special Ringer solution were the same as previously described (Mann, 1945*a, b, c*). Seminal vesicles from fully fertile rats were slit open along the outer edge and washed in Ringer solution to remove the secretory fluid. Portions of the basal parts of the organ (8–10 mg, dry weight) were used so as to include as much as possible of the 'coagulating gland', which in rats contributes, together with

the dorsal prostate, the bulk of seminal fructose. Seminal glands from bulls were collected from freshly slaughtered animals.

Methods. Three methods were used for the assay of fructolysis: (1) manometric estimation of acid production was carried out in Barcroft differential manometers with gas outlets, by measuring the CO_2 output using Ringer bicarbonate and a gas mixture of 95% N_2 and 5% CO_2 , (2) lactic acid was estimated by the method of Friedemann, Cotonio & Shaffer (1929), (3) fructose was determined colorimetrically as described before (Mann, 1948*a, b*). Respiration was measured manometrically in air and Ringer phosphate.

RESULTS

Utilization of fructose by sperm

Anaerobic fructolysis. Certain points which emerged from the study of fructolysis in washed spermatozoa are illustrated in Fig. 1. The experiment recorded in this figure was carried out with suspensions of washed spermatozoa of ram in Ringer-bicarbonate solution, incubated anaerobically by shaking at 37° in Barcroft manometers filled with 95% N_2 and 5% CO_2 . In each case the final volume of the mixture in the manometric flask was 2.5 ml. The following mixtures were used: I, 0.65×10^9 cells + 1.9 mg fructose, II, 0.65×10^9 cells + 1.9 mg glucose, III, 0.13×10^9 cells + 1.9 mg fructose. In I and II the acid formation followed the course of a straight line so long as there was still a little sugar left, after 110 min incubation the final yield of CO_2 was in I $420 \mu\text{l}$ CO_2 , corresponding to 1.68 mg lactic acid, in II $410 \mu\text{l}$ CO_2 , corresponding to 1.64 mg lactic acid. By chemical analysis of lactic acid, 1.63 mg was obtained in I, and 1.58 mg in II. Estimation of sugar showed that only 0.05 mg fructose was left in I, and 0.07 mg glucose in II. Taking the 60 min value as the basis for the calculation of the metabolic rate, one finds that $290 \mu\text{l}$ CO_2 or 1.16 mg lactic acid has been produced from fructose or glucose alike by 0.65×10^9 sperm cells contained in mixtures I and II, i.e. 1.78 mg/ 10^9 sperm/hr. However, in III the acid amounted to not more than $24 \mu\text{l}$ CO_2 / 0.13×10^9 sperm/hr, that is only 0.77 mg/ 10^9 sperm/hr.

Aerobic fructolysis and O_2 consumption Aerobic experiments were carried out with the same ram semen as above, but the suspensions of washed spermatozoa contained phosphate instead of bicarbonate, the amounts of sugar added were the same as before. The O_2 uptake in suspensions I and II can be seen to follow a linear course throughout the period of 110 min (Fig 2). However, whereas in the course of anaerobic activity (Fig 1), suspensions

sperm/hr. The low rate of respiration in III recalls the previously mentioned low rate of anaerobic

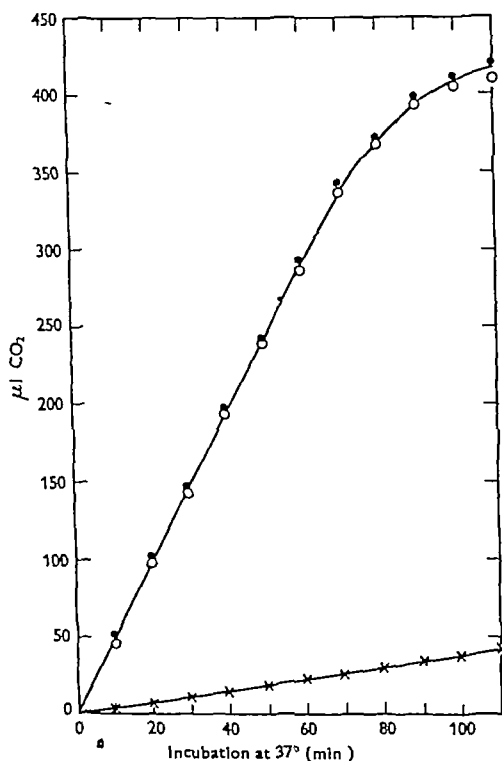


Fig 1 Anaerobic fructolysis and glucolysis by washed spermatozoa I ●—● 0.65×10^9 ram sperm + fructose, II ○—○ 0.65×10^9 ram sperm + glucose, III ×—× 0.13×10^9 ram sperm + fructose

I and II used up nearly all the sugar, in the corresponding aerobic experiment (Fig 2), out of 1.9 mg sugar added, 0.42 mg fructose and 0.48 mg glucose, respectively, were found to be still intact after 110 min. In the same samples lactic acid was determined, 1.03 and 0.98 mg lactic acid were found at the end of 110 min respiration period in I and II respectively. It can be seen from Fig 2 that the sperm suspensions I and II, each containing 0.65×10^9 sperm/2.5 ml Ringer solution, consumed $130 \mu l O_2$ in 1 hr or $200 \mu l O_2/10^9$ sperm/hr. However, suspension III which is five times more diluted than I and II (0.13×10^9 sperm/2.5 ml Ringer solution) consumed only $15 \mu l O_2$ or $115 \mu l O_2/10^9$

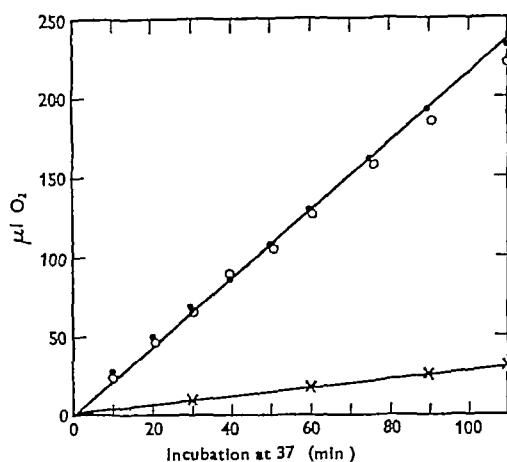


Fig 2 Oxygen consumption of ram spermatozoa I ●—● 0.65×10^9 ram sperm + fructose, II ○—○ 0.65×10^9 ram sperm + glucose III ×—× 0.13×10^9 ram sperm + fructose

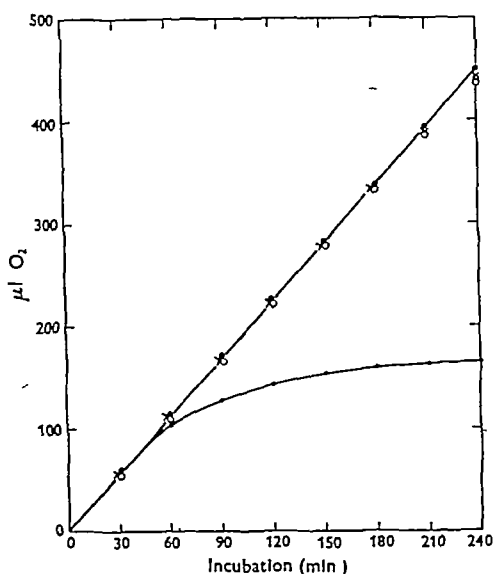


Fig 3 Effect of fructose, glucose and lactate on the respiration of washed spermatozoa 1 ml ram semen diluted with 3 ml. Ringer solution, centrifuged, sperm washed with 5 ml. Ringer solution, resuspended in Ringer solution and diluted to 16 ml. Each manometer flask contained 2 ml sperm suspension, corresponding to 0.45×10^9 sperm cells, and 0.5 ml isotonic phosphate buffer with — no additions, ●—● 1 mg fructose, ○—○ 1 mg glucose, ×—× 1 mg lactic acid (as Na lactate)

glycolysis and must be attributed to the same cause the injurious effect of dilution on spermatozoa

It has been known that ram spermatozoa freed from seminal plasma, and thus deprived of glycolyzable material, retain their ability to consume oxygen at an almost normal rate of respiration of about $200 \mu\text{l O}_2/10^9 \text{ sperm/hr}$ (Lardy, Winchester & Phillips, 1945, Mann, 1945 c) Thus the aerobic metabolism in spermatozoa appeared independent of fructolysis. However, in the present study, with suitable dilutions of washed spermatozoa, it was found that the O_2 uptake in such suspensions remained constant only for a limited period of time,

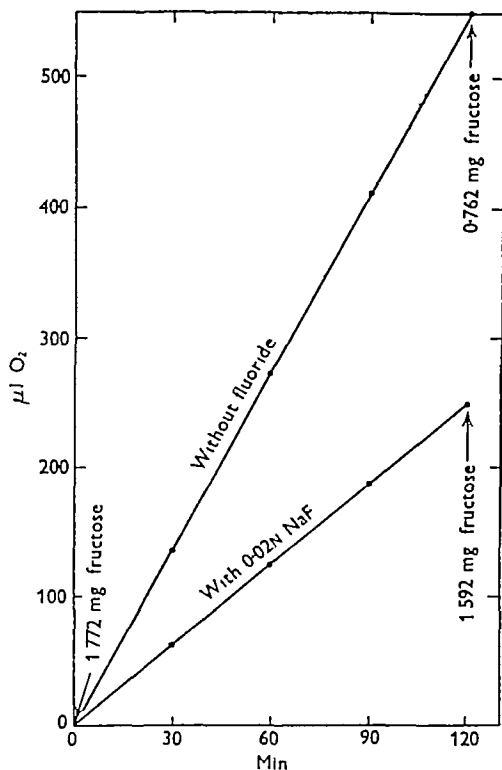


Fig. 4. Effect of fluoride on the respiration and aerobic fructolysis of ram semen. Each manometer flask contained 0.5 ml whole semen diluted with 1.5 ml. Ringer-phosphate solution.

and that during the later stage it declined progressively unless the sperm were provided with an additional source of oxidizable material such as fructose, glucose or lactate. Their effect on the O_2 uptake of washed spermatozoa is illustrated by Fig. 3, whence it can be seen that all three of them maintained equally well the initial rate of respiration for a considerable length of time, although they did not raise significantly the initial rate of O_2 consumption. On the other hand, if added to a respiring suspension of washed spermatozoa at a later stage, when the respiration had already begun to decline, they prevented further deterioration in the rate of O_2 consumption.

In order to explore the significance of glycolysis in the aerobic metabolism and survival of spermatozoa, use was made of certain substances such as iodoacetate and fluoride which were previously shown (Lardy & Phillips, 1943) to interfere with sperm activity. Iodoacetate proved to be a rather strong inhibitor not only of fructolysis but of the sperm respiration as well. More interesting results were achieved with fluoride. It was possible, using a suitable concentration of NaF, to suppress the fructolytic activity of whole semen much more strongly than the respiration. This can be seen from Fig. 4, which shows the O_2 uptake of two identical samples of semen, one treated with fluoride, while the other served as a control. In both these samples fructose was estimated at the beginning of the experiment and then after 2 hr aerobic incubation in Barcroft manometers at 37° . The results of fructose analyses are indicated by arrows on Fig. 4. It can be calculated from these values that 0.5 ml untreated whole semen consumed in 2 hr $550 \mu\text{l O}_2$ and 1.01 mg fructose, whereas the corresponding fluoride-treated sample utilized in the same period $250 \mu\text{l O}_2$, but only 0.18 mg fructose. When at the end of a 2 hr period the motility of the sperm was examined, the untreated spermatozoa were found to be perfectly motile, but those in the fluoride-treated semen were mostly immotile.

Fructose and glucose utilization in the seminal vesicle

It was shown in the foregoing that spermatozoa are cells capable of utilizing fructose and glucose both anaerobically and aerobically. In this respect they differ from most other animal tissues, the latter are able to metabolize fructose aerobically, but seldom show themselves capable of metabolizing it anaerobically at a rate comparable to that of glucose (Dickens & Greville, 1932, 1933). A study of carbohydrate metabolism in the seminal vesicle of the rat gave the following results, illustrated in Fig. 5. The anaerobic acid production in the seminal vesicles was found to be equally low in the absence as in the presence of added fructose. However, when glucose was added instead of fructose, it increased very strongly the rate of anaerobic glycolysis in seminal vesicles. It should be pointed out, however, that even the increased rate of glycolysis, as observed in the seminal vesicle in presence of glucose, was below the rate of glycolysis of spermatozoa. Assuming that the average rate of glycolysis in semen is 1.7 mg sugar or $425 \mu\text{l CO}_2/10^9 \text{ sperm/hr}$ and that the dry weight of 10^9 sperm is 30 mg, we arrive at the value of $Q_{\text{E}}^{\text{N}} = +14.2$ for the glycolysis quotient of spermatozoa. The seminal vesicle tissue, on the other hand, has been shown to produce anaerobically $2.3 \mu\text{l CO}_2/\text{mg dry wt/hr}$ and thus to have a quotient $Q_{\text{E}}^{\text{N}} = +2.3$.

In view of the marked differences in anaerobic metabolism which exist between the sperm cells and seminal vesicles the possibility was considered that similar differences may also prevail under aerobic conditions. Seminal vesicle slices incubated aerobically respired much better in presence of additional sugar, and the increase brought about by fructose was, if anything, even higher than that due to glucose (Fig 6). The Q_{O_2} values for rat seminal vesicle slices, calculated from the data of Fig 6, are -5.3 in presence of fructose, -4.2 in presence of glucose, and -2.0 in absence of added sugar. Even

of added carbohydrates. In presence of glucose, aerobically, a formation of free fructose was observed in slices, but the quantities were small. On the other hand, considerable quantities of 6-phosphofructose, together with 6-phosphoglucose, were formed from glycogen and Cori ester, by dialyzed and non-dialyzed extracts, but there was no increase in free fructose or lactic acid. It was also found that when dialyzed extract prepared from seminal vesicles was added to an actively glycolyzing Meyerhof muscle extract, it checked the formation of lactic acid from glycogen and led to the accumulation of 6-phosphohexose esters. It should

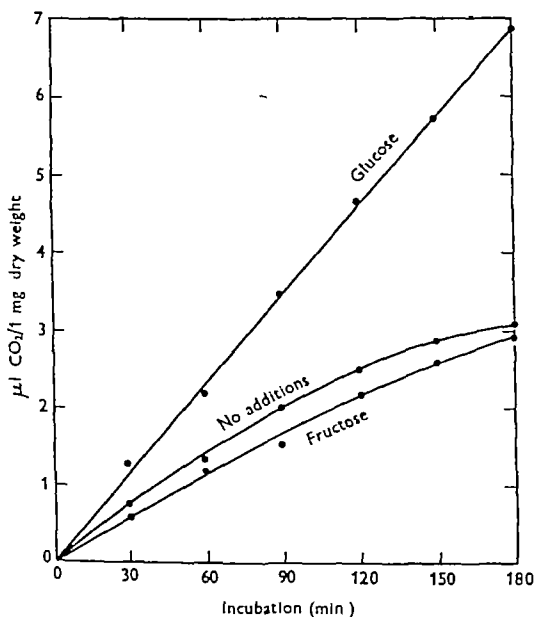


Fig 5 Anaerobic fructolysis and glucolysis in rat seminal vesicle

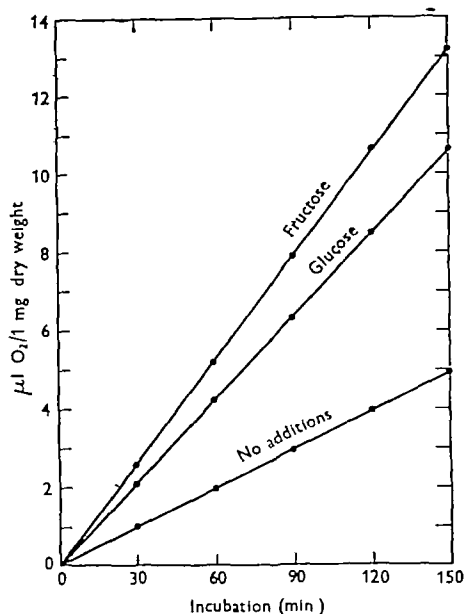


Fig 6 Respiration of rat seminal vesicle in presence of fructose and glucose

the highest of the three values is far below the Q_{O_2} of washed spermatozoa. Taking Fig 3 as the basis of calculations, the O_2 uptake of ram sperm amounts to $112 \mu l O_2 / 0.45 \times 10^9$ sperm/hr, i.e. $250 \mu l O_2 / 10^9$ sperm/hr. The dry weight of 10^9 sperm cells represents some 30 mg, so that the Q_{O_2} of spermatozoa is -8.3 .

Bull seminal glands were investigated in the following manner: the fresh gland was divided into two symmetrical portions, in one of which fructose was estimated at once, while the other was incubated at 37° for 2 hr. The level of fructose in the incubated sample did not differ markedly from the control. Similar results were obtained when the glands were minced or extracted with water and the pulp or aqueous extract used for incubation. A few experiments were also carried out in which minced seminal glands, slices or extracts, were incubated in presence

be pointed out, however, that the extracts from seminal glands contain very active phosphatases which dephosphorylate adenylic acid, adenosine triphosphate and coenzyme. Thus it is probable that the effects described above are at any rate partly due to the inactivation of the glycolytic coenzymes.

DISCUSSION

In bull and ram semen the anaerobic survival of spermatozoa is closely dependent upon the presence of fructose. If ram or bull spermatozoa are washed free from seminal plasma, and thus deprived of fructose, they soon become immotile under anaerobic conditions. But their survival can be extended considerably by the addition of glycolyzable sugar. So far, there is no evidence which would point to the existence of other anaerobic processes in semen,

Stage 5 The final precipitate of tropomyosin obtained by salting out to 70% saturation is dissolved in water, filtered through paper pulp, dialyzed at 0° for 2 days against many changes of distilled water (maintaining the pH inside the sac at 6.5), and finally against 0.1 M KCl. (The absence of free ammonium ions is checked by precipitating a portion of the sol with trichloroacetic acid and determining the N content of the filtrate.) The presence of KCl assists the subsequent precipitation with ethanol (effected with 1.5 vol.) The precipitate is dried through several changes of ethanol and then with ether. The dehydration of the protein is slow and is facilitated by pressing the gel particles on a sintered glass filter.

When the protein is required for re-solution, it may be stored (preferably at 0°) after drying as above, alternatively, it may be kept at 0° in the form of an $(\text{NH}_4)_2\text{SO}_4$ containing paste after a salting out procedure. When required for analysis, it is preferable to precipitate from a dialyzed sol by addition of dilute acetic acid to pH 5, such precipitates, after drying as before, contain only traces of ash.

Purity of the preparation The homogeneity of tropomyosin thus prepared was checked by electrophoresis (see below) and by ultracentrifugal analysis (Bailey, Gutfreund & Ogston, 1948). More rapid tests of purity, however, are available: (1) the glyoxylic reaction for tryptophan, using an equal weight of myosin as control, is extremely faint or negative, (2) a 2% sol in water (pH 6.5) is water-clear; any dispersed, denatured protein imparts an opalescence and tends to precipitate on heating in 0.1 M potassium chloride, (3) in absence or presence of salt (e.g. 0.5 M-potassium chloride), the protein dissolves in hydrochloric acid below pH 4 to give a clear solution.

Crystallization of tropomyosin The protein readily crystallizes, even from impure solution. A 2–3% solution (pH 6.5–7) is dialyzed at 0° against a salt solution containing 16 g ammonium sulphate/l and 0.01 M with respect to acetate buffer pH 5.4. When the pH of the sol reaches 5.8–6.0, crystals are deposited in large clusters on the cellophane membrane and drop into the bottom of the sac. The various forms observable are depicted in Pls 4 and 5 for skeletal rabbit muscle and pig cardiac muscle, but similar crystals have been obtained from teleost (whiting) muscle and from horse heart. In polarized light they are strongly birefringent, but in ordinary light they are difficult to view or to photograph because of their transparency. They are extremely fragile, and the larger crystals tend to break if stirred or touched.

In practice, it is often convenient to employ for crystallization the salted out precipitate described in the method of preparation. This is filtered off under gravity, allowed to drain overnight, mixed to a slurry with an equal volume of water, dialyzed (at constant volume) first against water until complete solution, then against several changes of the outer liquid described above. By retarding the rate of

change of pH inside the sac (either by using small volumes of outer liquid or infrequent changes of larger volumes) crystallization can be regulated to give large crystals.

Crystallization appears to depend upon the presence of sufficient salt to break up the large aggregates which are formed spontaneously in salt free solutions (see below), these unit molecules are then amenable to isoelectric crystallization. Crystals cannot be obtained either from pure water or from concentrated salt solutions. As a purification process, crystallization is quite inadequate, since tryptophan containing protein may still be detected in preparations three times crystallized. The composition of crystals obtained from material first purified by the methods already outlined, followed by three recrystallizations, appears identical with the original homogeneous, uncrystallized specimen (Table 1). Electrophoresis likewise gave proof of their homogeneity.

Table 1 *Composition of a three times crystallized rabbit tropomyosin compared with uncrystallized homogeneous tropomyosin*

	(Results* as percentage of protein weight)			
	Total N	Amide N	Tyrosine	Tryptophan
Homogeneous uncrystallized	16.7	0.89	3.2	c 0.02
Homogeneous crystallized	16.7	0.90	3.1	c 0.02
Unpurified crystallized	16.9	0.85	2.9	c 0.05

* See analytical section for methods of analysis

A quite unique property of the crystals is their enormous hydration. After sucking off excess mother liquor and pressing between filter paper, two different batches of crystals gave the following analysis:

Sample 1 water, 86.9, ammonium sulphate, 2.0, protein, 11.1%

Sample 2 water, 89.6, ammonium sulphate, 1.74, protein, 8.7%

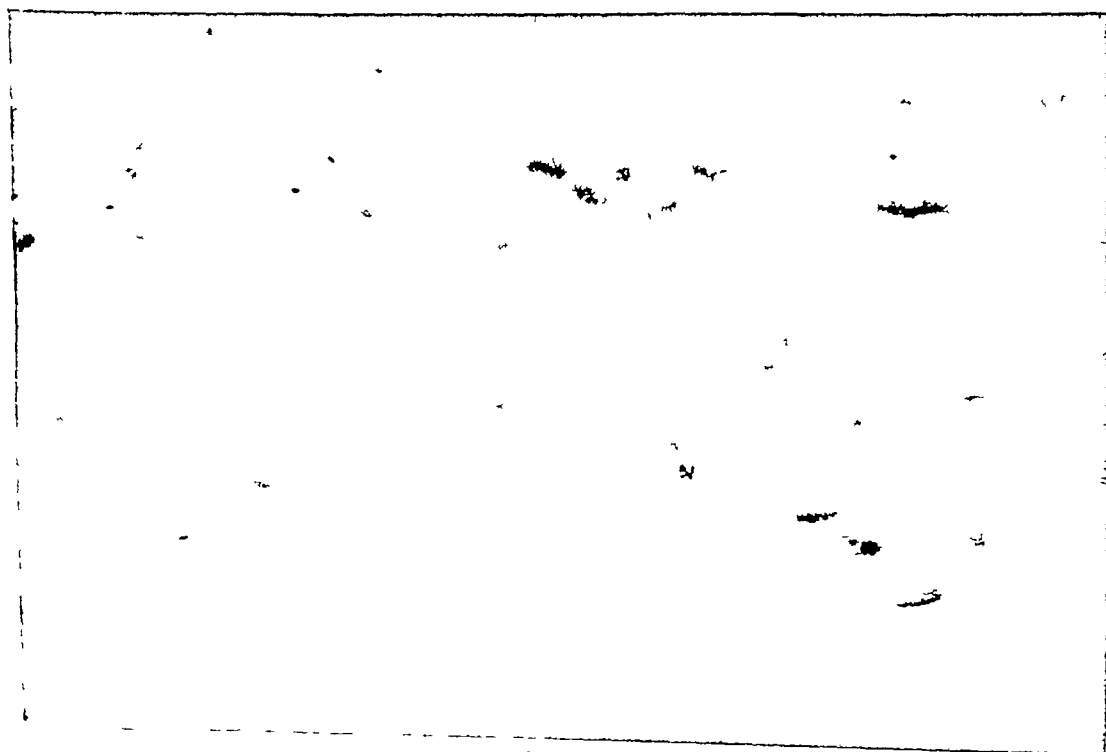
By comparison, lactoglobulin (McMeekin & Warner, 1942) contains 45.6 g and edestin (Bailey, 1942a) c 40 g of water/100 g of wet crystal.

Assay of tropomyosin

The method comprises essentially stages 1, 2 and 3 described in the preparation. The incorporation of certain modifications necessitates a résumé of the steps involved: (a) The minced muscle (100 g) is homogenized for 1 min. with an equal volume of water in a Waring Blendor, and squeezed out in muslin after addition of 200 ml ethanol. The residue is dried in ethanol and ether as before. (b) It is then extracted with 100 ml M KCl at pH 7, first for 12 hr at 15–20°, then for 3 hr, again for 3 hr, and washed finally with a further change of KCl solution. (c) The protein in the combined extracts is precipitated at pH 4.3 with N HCl, centri-



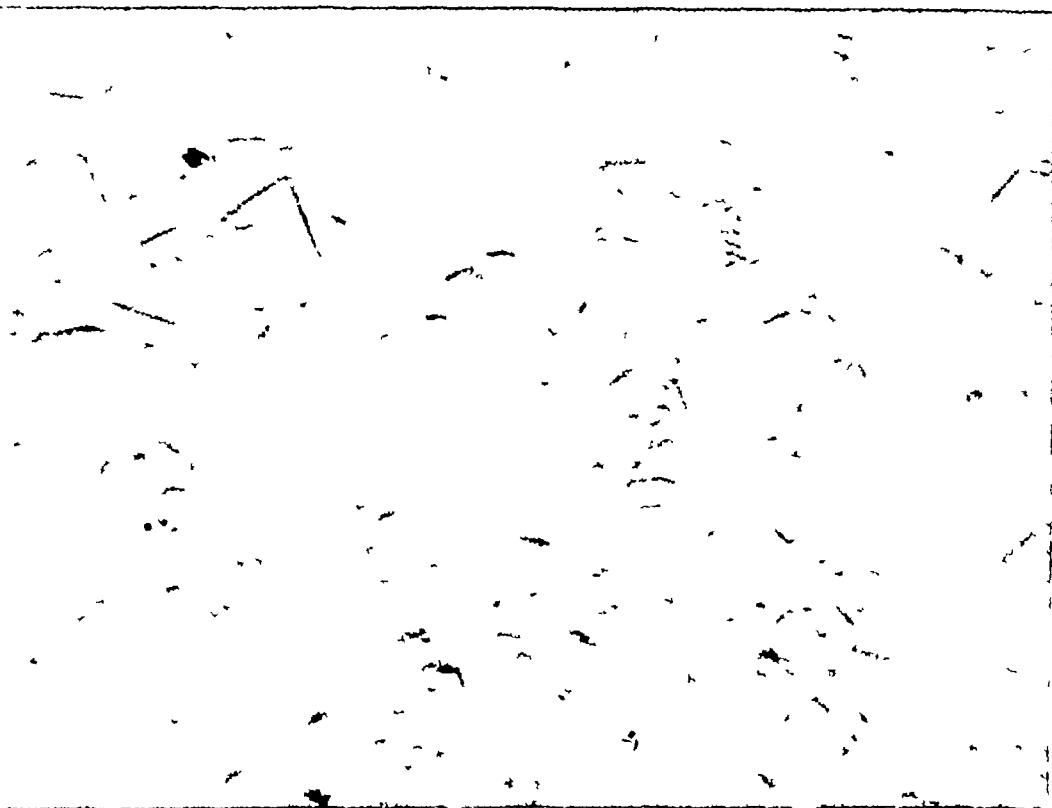
($\times 350$)



($\times 490$)

Tropomyosin crystals from rabbit skeletal muscle

K BAILEY—TROPOMYOSIN A NEW ASYMMETRIC PROTEIN COMPONENT OF THE MUSCLE FIBRIL



(a) ($\times 280$)



(b) ($\times 560$)

Tropomyosin crystals from pig heart muscle (a) ordinary light, (b) polarized light

fuged, the precipitate dispersed in water (50 ml) neutralized to pH 7 and treated with saturated $(\text{NH}_4)_2\text{SO}_4$ solution (containing 0.01 vol conc ammonia solution) to 41% saturation. The precipitate after centrifuging is saturated with 2 parts of 41% saturated $(\text{NH}_4)_2\text{SO}_4$ solution and again spun down. The combined supernatant fluids are treated with solid $(\text{NH}_4)_2\text{SO}_4$ to 85% saturation and the precipitated protein filtered off under gravity. It is then dissolved in 100 ml water and dialyzed for 4 days against frequent changes of 0.1M KCl solution. (d) To a 10 ml sample of the dialyzed sol is added 0.2 ml 1M phosphate pH 6.3, and the solution heated in a boiling water bath for 10 min. After cooling for 1 hr the slight precipitate of denatured protein is spun down leaving tropomyosin in solution. The protein content is determined by estimating the total N and correcting for free NH_3 by distilling an undigested portion of sol at pH 9.5. The protein N content $\times 6$ gives the yield of tropomyosin.

Prepared in this way the protein appears to be quite pure, the heat denatured impurity of step (d) for example gives a pronounced triptophan reaction whilst the tropomyosin isolated from the supernatant fluid only feebly responds.

The hind leg plus back muscle of adult rabbits gave yields of 0.42, 0.50, 0.50, 0.45, 0.46 g/100 g fresh muscle weight each value being obtained from a different animal. The mean value (0.47 g/100 g) comprises some 2.6% of the total protein and 4-5% of the myosin fraction. One determination on the cardiac muscle of the pig gave a lower value of 0.21 g/100 g muscle.

Tropomyosin as a naturally occurring constituent of muscle

The conditions for the preparation and assay of tropomyosin have evolved from a series of preliminary experiments directed towards the elucidation of three important points: (1) that the protein does not arise by post mortem catheptic breakdown, (2) that it is not produced by the disaggregating action of salt solutions upon ethanol denatured myosin, (3) that it is a constituent of the fibril and not of the sarcoplasm. These points will be considered in turn.

Tropomyosin and post mortem changes. Muscle tissue is characteristically poor in proteolytic enzymes (Fearon & Foster, 1922; Smorodinzew, Schirokow & Zyganowa, 1933), but even so, the exclusion of post-mortem changes was thought necessary. Homogenized muscle, obtained some 7 min after death, was divided into three portions, the first (A) was treated immediately with 1 vol ethanol squeezed and dried in the usual way, the second (B) was allowed to stand at 20° for 4 hr before addition of ethanol, the third (C) was washed immediately in three changes of water, and after standing for 4 hr was treated with ethanol. Tropomyosin was then estimated in all three residues.

The yields in all cases were quite comparable (Table 2). The arrest of proteolysis as in (A), or the removal of soluble enzymes as in (C), does not reduce the yield below that of (B) in which rigor changes are allowed to proceed.

Table 2 *Yield of tropomyosin from homogenized rabbit muscle*

Animal	Treatment of homogenate*	Yield of tropomyosin (g/100 g fresh muscle)
Rabbit DX	A	0.52
	B	0.47
	C	0.51
Rabbit FX	A	0.52
	B	0.48

* A, treated with ethanol 7 min after death; B, treated with ethanol 4 hr after death; C, washed thrice with water and treated with ethanol 4 hr after death.

The possible disaggregating effect of salt solutions in the production of tropomyosin. This second possibility was explored in two ways by studying the action of salts on (a) ethanol dried muscle, and (b) on ethanol dried myosin. In the first case it was shown that three successive extractions with 1M KCl suffice to exhaust the residue at a point where the total protein extracted is only some 4.5% of the myosin remaining in the residue, a fourth overnight extraction increased the yield by only 2%.

Likewise there is no evidence of disaggregation when myosin itself is treated in a similar manner. In this case, three precipitated myosins, prepared by the method of Bailey (1942b), was freeze dried and extracted with ethanol and ether. The residue (8.8 g) after three extractions with 1M KCl gave, in the fourth extract, a mere trace of material precipitable by trichloroacetic acid. The combined extracts yielded on appropriate treatment a small amount of material which, if tentatively considered as tropomyosin, amounted to 0.1% of the myosin taken. (It may be noted here that 1M KCl reduces the P content of dried myosin, due to the extraction of nucleic acid P.)

Tropomyosin as a component of the fibril. It has already been demonstrated (Table 2) that water extracted muscle mince is not depleted of tropomyosin, and that in consequence the histological site of the protein must reside either in the fibril, the collagenous supporting structures, or in the nuclear material. The evidence favours the idea that tropomyosin is of fibrillar origin. As will be shown later, tropomyosin must be considered, both chemically and structurally, a species of myosin, and is thus most likely to occur with myosin. Moreover, though a water soluble protein, it is extracted from muscle mince only under conditions which effect the dispersal of myosin (i.e. in 0.5-1.0M KCl), and then only slowly and incompletely. This dispersal is greatly facilitated by denaturing the fibrillar proteins in ethanol, and even then, salt solutions are necessary for extraction. In these phenomena we have a clear indication that *in situ* tropomyosin is fixed to the structural proteins, possibly by electrostatic forces arising from the large numbers of base and acid groups which it contains. The efficacy of the ethanol ether treatment might suggest in addition an association with lipids. Whatever the mode of combination, its liberation seems to depend upon a process of metathesis in presence of salt, a reaction simulative of that between histone and thymonucleic acid. For this reason, the conception of tropomyosin as a constituent of the nucleus cannot altogether be dismissed, but is improbable since the amount of thymonucleic acid, which may be

accepted as a guide to the total amount of nuclear matter, is only c 0.04–0.06% (Davidson & Waymouth, 1944, Schneider & Klug, 1946)

The data relating to some of these observations may now be given. In Fig 1 is shown the efficacy of water and salt solutions in extracting tropomyosin from ethanol- and ether dried muscle mince. The

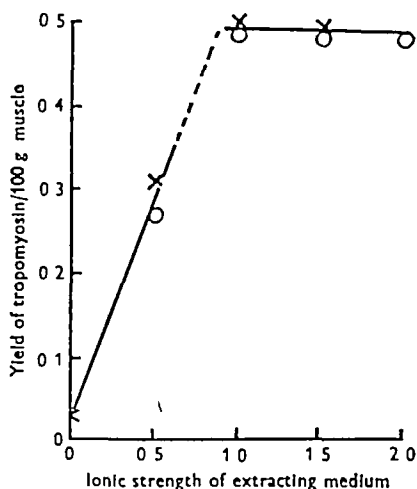


Fig 1 The yield of tropomyosin from minced, ethanol and ether dried rabbit muscle as a function of the ionic strength of the extracting medium (KCl). The two sets of points relate to two different rabbits

amount extracted by water is nil, and that by salt solutions a linear function of molarity up to 1.0 M, when the yield is maximal. If, on the other hand, freshly minced muscle is similarly extracted with salt solutions, the conditions here being optimal for extraction of myosin, the residues, after drying in ethanol and ether, still yield considerable amounts of tropomyosin, extraction with 0.5 M potassium chloride leaves 80% of the total, and with 1.0 M 20%, in the residue. The molarity of the salt solutions is again seen to be important in the case of fresh muscle, but even in 1 M potassium chloride, extraction is incomplete. The use of ethanol dried mince not only facilitates extraction, but gives an extract free from myosin and other soluble proteins. It may be noted that the acetone dried muscle residue used in the preparation of actin (Straub, 1942) still contains tropomyosin after the actin has been extracted.

Properties of tropomyosin

Solubility Above pH 6.5 and below pH 4.5, tropomyosin is soluble in water and in dilute salt solutions, the zone of complete insolubility in 0.01 M sodium chloride extends from pH 4.5 to 5.3. The rate of settling of the precipitated particles is most rapid at pH 5.1, indicating that the isoelectric point

lies near this pH value. In neutral ammonium sulphate solutions there exists a wide precipitation zone between the limits 45–60% saturation.

Denaturation In 0.1 M salt solutions, the protein is not precipitated by heating at 100° at pH > 6.3, the bulky precipitate which exists in the isoelectric zone transforms on heating to a discrete flocculum which again disperses at pH 7, not to a true solution, but to a translucent gel. After heating, or after dissolving in strong urea solutions, the protein no longer crystallizes. On the other hand, dehydrating solvents do not affect tropomyosin in any way, and the protein may best be stored by precipitating from its solution at pH 7 with ethanol, and subsequently drying in several changes of ethanol and then of ether. The protein is precipitated by trichloroacetic acid but redisperses at pH 6.5.

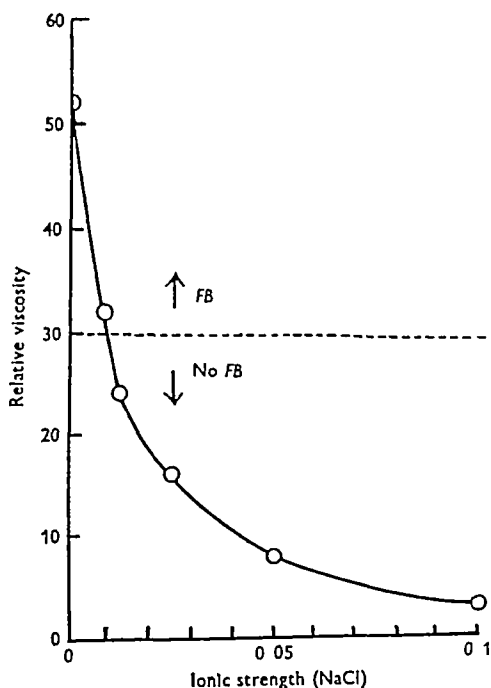


Fig 2 Relative viscosity of rabbit tropomyosin as a function of ionic strength (Ostwald viscometer). Protein concentration, 0.815%, pH, 6.5, temp 20°. Arrows indicate presence or absence of flow birefringence (FB) at low shear rates.

Viscosity In salt-free solutions at pH 6.5–7, tropomyosin forms viscous, non thixotropic solutions which show positive flow birefringence. On addition of neutral salts, there is a dramatic fall of viscosity between the ionic strength values $\mu = 0$ and 0.05, and at the latter value the flow birefringence at low shear rates just disappears. This behaviour is illustrated in Fig 2, which shows in addition that the lowest values of relative viscosity (e.g. 3.3 when $\mu = 0.1$, $c = 0.815\%$) are still high in comparison with

those of 'corpuseular' proteins. This high viscosity, even in salt solutions, first suggested that the molecule was inherently asymmetric, a conclusion fully supported by diffusion studies (Bailey *et al* 1945), X-ray studies (Astbury *et al* 1945) and by the high values obtained for the intrinsic viscosity. Again, the large increase in viscosity occurring after removal of salt appeared too large to explain except in terms of a polymerization into relatively large fibres, which were later seen in the electron microscope (Astbury *et al* 1948). This property was difficult to explain until the completion of the amino acid analysis, when it became clear that it was related, partly at least, to the high complement of dissociating groups which tend greatly to enhance the interaction of the protein with salt and of one protein molecule with another.

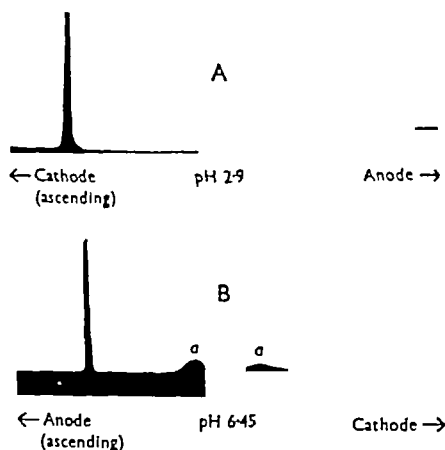


Fig 3 Electrophoretic patterns of rabbit tropomyosin (A) in 0.071M NaCl, 0.02M HCl, 0.08M glycine (pH 2.9 at 21°), protein concentration, 0.8%, 4°, overall voltage, 150 (B) in 0.05M phosphate, pH 6.45, otherwise, as for (A) but overall voltage, 200. Boundaries *a*, *a* are stationary and anomalous

Homogeneity The protein was submitted to electrophoresis in the Tiselus apparatus at pH 2.9 (glycine buffer) and pH 6.45 (phosphate buffer). The electrophoretic patterns both for the positively and negatively charged protein indicated a complete homogeneity (Fig 3). Although the ultracentrifuge data obtained by Dr A. G. Ogston (see Bailey *et al* 1948) likewise indicate a homogeneous substance, the crystals do not conform to a phase rule solubility. A possible explanation, which may also apply to some other proteins, is that the high water content of the crystals prevents the formation of a stable lattice, which can be so distorted during equilibration of the crystals that ultimately an amorphous product may be produced with a different solubility.

Amino acid analysis of rabbit tropomyosin

The homogeneous sample of protein which had been submitted to sedimentation and electrophoretic experiments was used for the assay of individual amino acids. Hydrolysis was effected by refluxing with constant boiling HCl (10 ml/g protein) for 24 hr. Excess HCl was removed by repeated evaporation *in vacuo*. In the determination of some components (total N, amide N, P, S, carbohydrate, hexosamine, cysteine + cystine, methionine, tryptophan, tyrosine) hydrolysis was carried out by means specific to the methods cited below.

Methods. Total N (Chubb, Rees & Williams, 1943) after drying *in vacuo* at 100° over P₂O₅, total P (Allen, 1940), carbohydrate (Pirie, 1946), hexosamine (Palmer, Smyth & Meyer, 1937), total S by alkali fusion (Lugg, 1938), cyst(e)ine and methionine by differential oxidation (Lugg, 1938), tryptophan and tyrosine (Lugg, 1937), amide N by partial hydrolysis at 37° in conc. HCl for 6 days (see Rees, 1946). Basic amino acids were kindly determined by Dr H. T. Macpherson (Macpherson, 1946), the lysine N, obtained as the difference of catholyte N and histidine plus arginine N, agreed well with the value obtained by Dr E. F. Gale employing his manometric decarboxylase method (Gale, 1947). Serine and threonine were determined by Mr M. W. Rees using a modification (Rees, 1946) of the Nicolet & Shinn (1939) method; the figures were corrected for destructive losses during hydrolysis. The monoaminomonocarboxylic acids were assayed by partition chromatography by Dr G. R. Tristram. These values are approximate, since amino acid recoveries were not checked with the appropriate control mixture (Tristram, 1946). The dicarboxylic amino acids were quantitatively isolated from 3 g. of protein by the Foreman (1914) lime ethanol procedure as adapted by Bailey, Chubb, Rees & Williams (1943). Glutamic acid was independently estimated by Dr E. F. Gale by his decarboxylase method. In the isolation of the dicarboxylic acid derivatives, detailed below, an approximate assay of glycine was obtained from a fraction freed from the bases, dicarboxylic acids and a large part of the less soluble monoaminomonocarboxylic acids. Two methods were used: (1) the trioxalatochromate method of Bergmann & Niemann (1937-8), and (2) the chromatographic, now made possible by the absence of the bases and dicarboxylic acids. Both methods revealed so little that the presence of glycine is considered doubtful.

Glutamic and aspartic acids by isolation. The Ca salts were prepared from a sample of hydrolysate containing 0.4683 g. N under the conditions outlined by Bailey *et al* (1943) for ovalbumin. The initial removal of cyst(e)ine with Cu₂O was omitted since the content of this amino acid is small. The steps in the analytical procedure may be briefly enumerated and compared with the quantitative data of Table 3: (1) After two volume ethanol precipitations, the ethanol soluble amino acids (A) were reserved, the Ca salts (B) were decomposed, yielding in the order of isolation, crops GA₁, GA₂ of glutamic acid HCl, AA₁ of Cu aspartate and GA₃. After a third lime ethanol precipitation of the final mother liquor, the Ca salts yielded GA₄, AA₂, GA₅ and GA₆. Each crop was recrystallized and the mother liquors added to the main filtrate before proceeding to subsequent isolations. (2) The ethanol soluble salts (A) were neutralized to pH 7 with oxalic acid, Ca oxalate removed, and the filtrate evaporated to a small volume *in vacuo*, giving a crystalline monoamino

acid fraction (C) The mother liquor was then treated with Na free phosphotungstic acid under the conditions recommended by Van Slyke, Hiller & Dillon (1942) The phosphotungstates of the basic amino acids were recrystallized and discarded, and phosphotungstic acid removed from the combined mother liquors The final solution of monoamino acids was freed from inorganic ions Cl^- by Ag_2O in presence of H_2SO_4 , Ag^+ as crystalline sulphate and as Ag_2S , SO_4^{2-} by baryta, Ca^{++} by oxalic acid The filtrate was evaporated when a further crop of monoamino acids crystallized The residual liquor was retained for the determination of glycine

Table 3 *Dicarboxylic acids and glycine in rabbit tropomyosin*

(Amino acid N as percentage of total N)

N in Ca salts (2 precipitations)	29.6
Crops GA_1 - GA_6 of glutamic acid HCl	17.6*
(Glutamic acid by decarboxylase method)	18.7)
Crops AA_1 - AA_2 of Cu aspartate	5.74*
Manipulative losses	0.1
Glycine (trioxalatochrome method)	0.5
Glycine (chromatographic method)	1.1

Analysis Glutamic acid HCl Found N, 7.64% Calc N, 7.63% $[\alpha]_D + 29.4^\circ$, indicating admixture with 3.4% of the D acid Cu aspartate (water free basis) Found N, 7.27, Cu, 32.0 Calc N, 7.19, Cu, 32.0%

* Solubility corrections (Bailey *et al* 1943) applied

Analytical characteristics of tropomyosin In Table 4 are collected the N, P, S and carbohydrate content of tropomyosin and myosin, together with evaluations of those amino acids determined by two different methods In Table 5, amino acid values are converted to g residues The source of data for myosin has already been published (Bailey, 1944), but revised values for the basic amino acids (Macpherson, 1946) are now included The analysis of myosin is lacking in respect of the dicarboxylic acids, since the titration data of Dubuisson & Hamoir (1943) indicate a higher content than that found by an older isolation method It must also be remembered that specimens of myosin hitherto analyzed contain a combined adenosine triphosphatase, not yet proved to be an integral part of myosin itself, and some 1-2% actin (Perry, unpublished) With both myosin and tropomyosin, chromatographic methods are approximate but comparative In the values listed, the second place of decimals is not a guide to the precision of the methods, where results are obtained initially on a weighed quantity of protein, the second place is retained on conversion to a N/N basis (and vice versa) for the sake of averaging

It will be seen (Table 4) that tropomyosin contains no appreciable amount of P or carbohydrate Amongst the amino acids, tryptophan is entirely lacking and glycine probably so

On comparison with myosin (Table 5) it is at once striking that the amounts of the monoamino mono carboxylic acids, methionine, tyrosine, alanine,

valine, leucines, phenylalanine, serine and the imino acid proline, are of comparable magnitude Both proteins are rich in arginine, lysine and glutamic acid, though the amounts vary from one protein to the other The data in no way suggest that the two proteins are chemically identical, or even that they might be if myosin were freed from probable contaminants There are large disparities in at least four constituents which are capable of precise assay threonine, histidine, tryptophan and amide N

Table 4 *Analysis of rabbit myosin and tropomyosin*

	Tropomyosin (as % protein wt)	Myosin (as % protein wt)
Total N	16.6-16.7	16.6-16.8
P	0.02	0.04-0.06
Organic S	0.80	1.10
Glucosamine	Nil	Nil
Total carbohydrate	<0.2	<0.2
	(As % protein N)	(As % protein N)
Lysine N (D)	17.6	13.6
Lysine N (E)	18.0	13.8
Glutamic acid N (I)	17.6	>12.6
Glutamic acid N (E)	18.7	—

D, N difference, E, enzymic, I, isolation methods (see text)

The amount of any one amino acid has so little significance as yet in the interpretation of structure and biogenetic relationships, that it is most profitable (Astbury, 1942, Bailey, 1944) to compare whole groups of amino acids expressed as a percentage of the total residues This gives a precise characterization of the protein in terms of types of side chains In both myosin and tropomyosin, the amino acids with non-polar side chains (methionine, tryptophan, phenylalanine, valine, leucines, proline, alanine, glycine) comprise c 35% of the total, the polar side chains are calculated as 63% for tropomyosin and 57% for myosin, the latter figure being minimal on account of analytical deficiencies (see above) The high valence of both proteins is characteristic, and in terms of charges of both negative and positive type, the value of 45% for tropomyosin is higher than in any other protein yet analyzed The corresponding value for myosin is 34, fibrinogen 26, edestin 27, β lactoglobulin 28, casein 23, insulin 23 (including end groups), ovalbumin 20 and zein 3% (It will be noted that the globulins fall in the same group by virtue of their high valence)

Though this type of comparison again reveals differences between the two proteins, it shows in respect of the most characteristic feature of the myosin molecule, viz the high content of base and non amidized carboxyl groups, that tropomyosin is a protein of myosin type

Table 5 *Analysis of rabbit myosin and tropomyosin*

(Results calculated on N contents of 16.7%)

	Tropomyosin			Myosin		
	N as % protein N	Wt./100 g	Residues/100 g	N as % protein N	Wt./100 g	Residues/100 g
Cystine/2	0.53	0.76	0.0063	0.98	1.4	0.0117
Methionine	1.58	2.8	0.0188	1.91	3.4	0.0228
Tyrosine	1.44	3.1	0.0172	1.58	3.4	0.0188
Tryptophan	Nil	Nil	—	0.66	0.8	0.0039
Glycine	(0.5)	(0.4)	—	2.1	1.9	0.0253
Alanine	8.3	8.8	0.0988	6.1	6.5	0.0730
Valine	2.25	3.13	0.0267	1.85	2.58	0.0221
Leucines	10.0	15.6	0.1190	10.0	15.6	0.1190
Phenylalanine	2.35	4.6	0.0270	2.2	4.33	0.0262
Proline	0.95	1.3	0.0113	1.4	1.92	0.0167
Serine	3.50	4.38	0.0417	3.46	4.33	0.0412
Threonine	2.04	2.90	0.0244	3.61	5.11	0.0429
Histidine	1.38	0.85	0.0055	3.9	2.41	0.0155
Arginine	15.0	7.8	0.0448	14.2	7.36	0.0423
Lysine	18.0	15.7	0.1074	13.7	11.92	0.0814
Glutamic acid	18.7	32.9	0.2236	12.6	22.1	0.1503
Aspartic acid	5.74	9.1	0.0684	5.6	8.9	0.0669
Amide N	5.35	0.89*	0.0636	7.19	1.20*	0.0857
Totals	97.11	113.72	0.8418	93.04	103.96	0.7800
Average residue wt		Tropomyosin			Myosin	
(1) From N partition		115.6	Mean 116.4	115.8	Mean 115.5	
(2) From wt. of residues		117.2		115.3		
Residues/10 ⁵ g protein		859		860		
As % of total residues						
Free acid groups		26.6	Total 45.0	18.0†	Total 34.1	
Base groups		18.4		16.1		
Non polar groups		35.2		35.7		
Polar groups		62.8		57.2		
Hydroxyl groups		9.7		11.9		
Amide groups		7.4		9.9		

* Not included in the summations, since the amidized dicarboxylic acids have approximately the same molecular weight as the non amidized.

† In the calculation for myosin the free acid groups found by Dubuisson & Hamoir (1943) from titration data (0.156 residues/100 g) have been used.

DISCUSSION

The present research has attempted a general survey of the properties of tropomyosin rather than a detailed study of any one aspect. Although the main interest must centre upon the role of the protein in the fibril, it should be noted that from the standpoint of its asymmetry and homogeneity, tropomyosin possesses an independent importance as an ideal molecule for the test of hydrodynamical theory.

There is no direct evidence to define the role of tropomyosin, though there are pointers as to its nature. All evidence suggests that it is a prototype of myosin. It is a very elongated molecule (Bailey *et al.* 1948, Astbury *et al.* 1948), capable of electrostatic interaction into relatively enormous fibres of rather regular width and 3000–6000 Å in length. Its solubility properties are similar to those of myosin,

particularly in respect of the pH insolubility zone, its solubility in acid solutions, and the lack of visible denaturation effects when such solutions are neutralized. Like myosin, it possesses a globulin-like solubility, for it not only has a limited solubility in dilute salt solutions on the alkaline side of the isoelectric zone (from pH 6 to 7), but the salting in effect of neutral salts on tropomyosin crystals is quite marked. Again, the overall characteristics of the amino acid pattern are of myosin type, and there are striking similarities in the absolute amounts of many individual amino acids, more, in fact, than might be expected to arise by mere coincidence. At the same time, there are divergences which refute the idea that myosin and tropomyosin are chemically identical. Lastly, tropomyosin, like myosin, gives a well defined large angle X ray pattern of a type (Astbury *et al.* 1948), and is the first member of the keratin myosin epidermis fibrinogen group to be both genuinely fibrous and genuinely crystalline.

In the light of these properties it is not unreasonable to suggest that tropomyosin may be one of the ultimate units of which myosin is composed (cf Bailey 1946*b*). *In situ*, it seems to be firmly bound to the substance of the fibril, and requires methods of extraction which suggest that the process is one of metathesis. Its molecular weight of 90,500 (Bailey *et al.* 1948), moreover, is quite comparable with that of myosin after depolymerization in urea solutions, a process which is no longer assumed to involve anything more than the rupture of secondary valence bonds (Mirsky & Pauling, 1936), these subunits of myosin have an average molecular weight c. 10^5 (Weber & Stöcker, 1933). If in some way the protein is built up into the myosin filament, it might be misleading to cite the spontaneous electrostatic interaction of tropomyosin molecules as being the precise means of aggregation *in vivo*. It demonstrates rather the ability of the molecules to aggregate in regular fashion, whatever the mechanism employed in nature.

Although the hypothesis cannot be enlarged in point of detail, since it concerns an aspect of protein chemistry quite unknown and unexplored, it is a logical extension, however tentative its nature, of the general principles which seem to govern the structure of proteins. In their elaboration there are at least three levels of molecular organization: the first concerns the intramolecular pattern, the second, the aggregation of polypeptide chains (themselves dissimilar in the case of insulin (Sanger, 1945)) into submolecules, and the third, the aggregation of these latter units to give the native protein molecule. If differences exist in the elaboration of a fibrous protein as compared with a corpuscular, they are probably differences of degree rather than of kind, and if it is true that the depolymerization effects observed outside the pH stability zone, or in urea and guanidine solutions, are not due to the cleavage of covalent linkages, then it follows that the stability of the whole molecule is a reflexion only of the number and types of secondary valence forces. Such a generalization appears to be true, at least, of proteins like myosin, in which interchain disulphide bridges are not operative, but even where these do occur, they seem to be utilized in the formation of a stable unit (as in the case of insulin), the aggregation or disaggregation of units being a reversible process involving only secondary valence forces.

A possible, though by no means the only, mechanism (compare, for example, Astbury *et al.* 1948) that can be visualized in the elaboration of a myosin filament is that the necessary cohesion derives from the union of units with specific, interlocking side chain patterns. Whether we seek the explanation of enzyme action, antigen antibody formation, or the nature of the unusual colloid interaction of myosin and actin (Bailey & Perry, 1947*a, b*), we are led to this conception of specificity as a fundamental property of the protein molecule. It is not illogical, therefore, to invoke the same property as responsible, perhaps, for the elaboration of a native protein from its component units. It is almost

inconceivable that a myosin (actomyosin?) filament of some 15,000 Å in length (Hall, Jakus & Schmitt, 1946) could arise fully fashioned from an enzyme template, or that its biogenesis derives from the unfolding of already folded units (cf Lawrence, Muir, Needham & Shen, 1944). It could be conceived more readily as a process, an autocatalytic process even, requiring only the presence of units with interlocking side chain patterns. Such units may not be identical, and it is certain, at least, that myosin consists of something more than tropomyosin units.

The experimental verification of the role suggested for tropomyosin is obviously one of great difficulty, but indirectly it would receive strong support if other types of units (cf Astbury, 1947) from other fibre systems (e.g. the collagens and keratins) are eventually isolated.

SUMMARY

1 A new asymmetric, homogeneous protein, termed *tropomyosin*, has been isolated from skeletal and cardiac muscle.

2 Though water soluble in neutral solutions after isolation, it can be extracted from the tissue only by salt solutions, and most easily from ethanol and ether dried tissue, it thus appears to be firmly attached to the structural components of the fibril.

3 In dilute salt solutions, tropomyosin crystallizes in large birefringent plates containing only 9–11% of protein. In absence of salt, solutions of the protein are exceedingly viscous, due to the aggregation of molecules by electrostatic interaction into large fibres.

4 In rabbit skeletal muscle it occurs to the extent of 0.5 g/100 g of wet muscle.

5 The isoelectric point is near pH 5. Its solubility is little affected by heat treatment or by depolymerizing agents such as urea, although the ability to crystallize is lost. On the alkaline side of the isoelectric point, the protein may be dried in organic solvents without loss of solubility.

6 Its amino acid composition is of myosin type, and like myosin, it gives an X-ray diffraction pattern of α -type.

7 The properties of tropomyosin suggest that it is a prototype of myosin, and the possibility exists that it is one of the units from which the myosin filament is elaborated.

I am much indebted to the various colleagues already mentioned for the application of methods in which they are skilled, to Prof A. C. Chibnall, F.R.S., Mr G. S. Adair, F.R.S. and Dr E. C. Bate-Smith for helpful discussions, to Dr A. G. Ogston for reading the manuscript, lastly and especially to Prof W. T. Astbury, F.R.S. for his inspiration.

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Molecular Weight of Tropomyosin from Rabbit Muscle

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In the preceding paper (Bailey, 1948) the preparation and properties of tropomyosin are described. The present paper deals with the determination of its molecular weight by three methods: osmotic pressure, sedimentation-diffusion and amino acid analysis. The partial specific volume and density of the dry protein have also been determined.

METHODS AND RESULTS

Tropomyosin samples Two preparations have been examined: sample A, identical with that used for amino acid analysis and shown to be electrophoretically homogeneous (Bailey, 1948), and sample B for which there were only analytical criteria for purity.

Osmotic pressure Measurements were carried out at 0° by the method of Adair (1925) in buffer of composition 0.2M KCl, 0.0133M Na₂HPO₄, 0.0267M NaH₂PO₄, pH 6.5. Protein concentration was determined by the micro

Kjeldahl method, taking 16.7% as the N content of the protein.

Following the procedure of Adair & Robinson (1930), the ratio of osmotic pressure (cm of water) to protein concentration (P/C) is plotted against C (Fig. 1), concentration is expressed as g/100 ml solvent, and is obtained by employing the determined partial specific volume (see below) of 0.71. It will be seen that P/C varies considerably with C , whereas for some proteins (e.g. lactoglobulin, ovalbumin, serum albumin) the variation is small over a similar range of concentration, provided that C is expressed as g/100 ml solvent. This anomaly is probably due either to a thermal interaction between the particles or to a statistical interaction due to their asymmetry. Extrapolation of P/C to zero concentration gives a value of 2.63, indicating a molecular weight of 88,000.

Sedimentation-diffusion data The sedimentation constant was obtained by examination in the Svedberg oil turbine ultracentrifuge at Oxford using the method of Philpot (1938). The concentrations of the protein were determined refractometrically, assuming a specific refractive increment of 0.00180, the solvent was as above, but contained NaCl instead of KCl. The speed was 1010 rev/sec

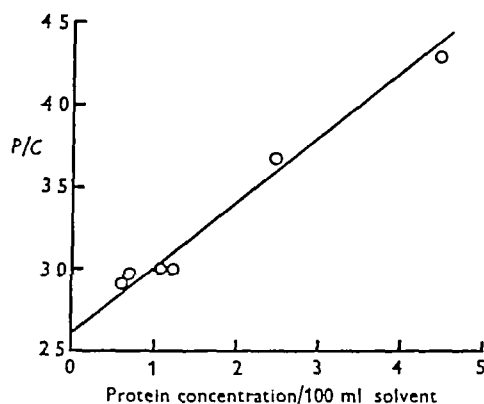


Fig 1 Osmotic pressure/protein concentration (P/C) as a function of C (rabbit tropomyosin) P in cm water, C in g/100 ml. of solvent Buffer 0.2M KCl, 0.0133M Na_2HPO_4 , 0.0267M NaH_2PO_4 , pH 6.5, 0°

The diffusion constant was determined by two different methods. In method 1, measurements were carried out in a cell similar to that of Lamm & Polson (1936) and boundaries were observed by the Philpot (1938) optical system. The diagrams were enlarged photographically and the diffusion constant D calculated from the formula $D = \sigma^2/2t$, where σ represents half the distance between inflexion points, and t the time in sec. The position of inflexion points was located by dividing the maximal height of the curve by \sqrt{e} . Two runs were carried out, one at 0.7% protein concentration and one at 1.2%, the solvent was that of the osmotic pressure measurements. Four photographs of the boundary were made during each run, and mean values of D were derived from the four curves of each experiment. Method 2 is essentially new and is described elsewhere (Coulson, Cox, Ogston & Philpot, 1948).

The sedimentation diagram for sample A (Fig 2) showed a single homogeneous component of $s_{20}(\text{corr})$ 2.60×10^{-13} . Integration of the areas of the boundary in the diagram gave 103% recovery of the refractive increment (Philpot, 1939, Johnston & Ogston, 1946), showing that this boundary includes the whole of the sedimenting material. Sample B was likewise homogeneous.

The several values of s and D corrected to 20° and to a water basis are given in Table 1, molecular weights were calculated by the usual formula

$$M = \frac{RTs}{D(1 - \bar{v}\rho)}$$
 using a value of 0.71 for the partial specific volume (\bar{v}). The mean value from these data is 92,700, somewhat higher than that by osmotic pressure. It should be noted, however, that there is a marked variation of D (and probably also of s) with

concentration, strictly, the molecular weight should be estimated from the extrapolated values at zero concentration.

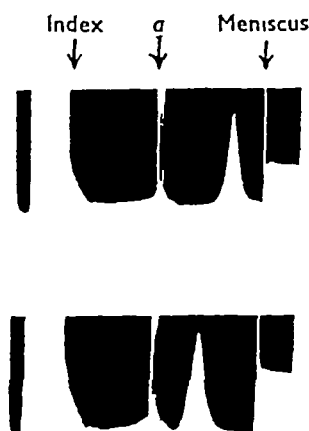


Fig 2 Sedimentation diagrams of rabbit tropomyosin, 0.6%, 35 and 65 min. after reaching full speed. The shadow marked *a* is due to aberrant cell washer, which did not interfere with the sedimentation process.

Table 1 Molecular weight of rabbit tropomyosin from sedimentation diffusion data in solutions $\mu = 0.267$

Sample*	Protein concentration (g/100 ml)	$s_{20}(\text{corr}) \times 10^{13}$	$D_{20}(\text{corr}) \times 10^7$	Mol wt
A	1.20	—	1.78	—
	0.70	—	2.32	—
	0.60	2.60	(2.43)†	89,500
B	0.665	2.51	2.22	94,500
	0.635	(2.55)‡	2.26	94,300
Mean 92,700				

* D for sample A by method 1, sample B, method 2 (see text).

† By extrapolation of values for 1.2 and 0.7% protein concentration to 0.6%.

‡ By interpolation of values for 0.60 and 0.665% protein concentration.

Molecular weight from the histidine content Since histidine may be determined accurately (Macpherson, 1946), and since the amount in tropomyosin (0.85 g/100 g protein) is sufficiently small, the minimal molecular weight multiplied by some small whole number should lead to a reliable value for the true figure. The minimal molecular weight thus obtained (18,180), multiplied by factors of 4, 5 and 6, gives the values 72,700, 90,900 and 109,100 respectively. Of these, only the middle value approaches those found by other methods.

Mean molecular weight The mean molecular weight, derived from osmotic pressure, sedimentation diffusion (mean value) and analysis, is 90,500. Using this value and an average diffusion constant of 2.35×10^{-7} (probable value at 0.6% protein concentration), D_0 , the diffusion constant of a spherical

molecule of similar molecular weight, is found to be 7.2×10^{-7} , giving a frictional ratio $D_0/D = f/f_0 = 3.1$. This value, as far as can be ascertained, is much greater than any recorded for a protein of comparable molecular weight, though ideally it should be calculated from the value of D at zero concentration. As in the case of other proteins, the calculation of asymmetry from the frictional ratio is complicated by the uncertain magnitude of the water of hydration. Taken in conjunction with physical and X-ray evidence (Bailey, 1948; Astbury, Reed & Spark, 1948), the high value of f/f_0 indicates in a qualitative manner the pronounced asymmetry of the molecule.

Partial specific volume and density of dry protein. Determinations of partial specific volume (\bar{V}) have been made both in water and in salt solutions, using 25 ml density bottles equilibrated at $20.8^\circ \pm 0.1$. Corrections were applied for buoyancy and for the small amount of ash in the protein. In salt-free medium the viscosity is so high that it was necessary to evolve a special technique: the isoelectric protein (dried in ethanol, ether and *in vacuo*) was weighed into the bottle and a calculated volume of $\sqrt{70}$ NaOH added to give a final pH of 6.5. Within 24 hr the protein had swollen to a viscous sol from which air bubbles were removed by light centrifuging. Distilled water was now added to capacity and the stopper inserted. Since there was no admixture of protein with the upper water layer, the loss of liquid in this latter operation does not incur loss of protein. A correction was applied for the contribution of Na ions to the density of the medium.

In salt solutions an accurate salt concentration was obtained by adding either NaCl or K_2SO_4 to a dialyzed sol and diluting to 100.0 ml. Samples were then transferred to the density bottle. Protein concentration was determined both by dry weight and by N content, and experiments in which values disagreed by more than 1% were discarded. All samples were measured by weight and not by volume.

The mean value of \bar{V} (Table 2) is 0.71, that calculated by summation of amino acid residues listed in the previous paper (Bailey, 1948) is 0.735. Because of this discrepancy the details for the determination of \bar{V} have been given at length. It seems clear that the assumption of \bar{V} in the calculation of molecular weights may on occasion give rise to serious error, though it is true that in other cases the calculated values of \bar{V} agree well with the observed (Cohn & Edsall, 1943). It is possible that the dis-

crepancy is confined to proteins with large amounts of acid and base groups, and due either to an intense electrostriction of the molecule itself, or, more probably, to electrostriction of the water of hydration.

Table 2 *Partial specific volume (\bar{V}) and density (ρ) of dry tropomyosin*

Medium	\bar{V}	ρ
Water	0.708	—
Water	0.703	—
NaCl (1.0 M)	0.704	—
K_2SO_4 (0.064 M)	0.715	—
Paraffin	—	1.270
Paraffin	—	1.278
Xylene	—	1.280
Mean	0.71	1.28

The density of tropomyosin after drying at 100° *in vacuo* over P_2O_5 is given in Table 2. In these experiments, measurements were carried out using paraffin or xylene as displacing medium, removing entrapped air bubbles from the protein by evacuating after immersion of protein in the medium. It will be noted that there is a very large discrepancy between the 'apparent density' of the protein in solution ($1/0.71 = 1.41$) and the determined value for the dry protein (1.28). Similar discrepancies, though not so large, exist in other proteins; the density of dry lactoglobulin (McMeekin & Warner, 1942) is 1.26 and the 'apparent density' from the value of $\bar{V} = 0.754$ (Podersen, 1936) is 1.33.

SUMMARY

1 Tropomyosin from rabbit skeletal muscle is entirely homogeneous in the ultracentrifuge.

2 The molecular weight in salt solutions ($\mu = 0.267$) is found to be 88,000 by osmotic pressure and 92,700 by sedimentation diffusion; the value derived from the histidine content is 90,900 and the mean of all values, 90,500.

3 The osmotic pressure and diffusion constant (and thus probably the sedimentation constant) are markedly dependent upon protein concentration. At the lowest concentration investigated (0.6%), the frictional ratio is 3.1, indicating a very asymmetric molecule.

4 The partial specific volume (0.71) is lower than that calculated from amino acid residues (0.735).

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An X-ray and Electron Microscope Study of Tropomyosin

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METHODS AND RESULTS

X rays

A technique that we have found convenient in trying to obtain oriented preparations of elongated macromolecules is to make a thin film by drying a pool of sol on a glass plate, and then to stretch narrow ribbons of the film (Astbury & Dickinson, 1940). The first step tends to leave the molecules lying parallel to the surface of the film but in all azimuths, while the second tends to draw them parallel also to the direction of stretching, so that in the end an X ray fibre diagram should result. Even without the second step, however, an imperfect fibre diagram may be expected if the specimen is photographed with the X ray beam parallel to the surface of the film. Further orientation and stretching effects may be produced by squeezing the film between small pieces of plate glass clamped together by screw clips.

Thin films of tropomyosin are readily prepared from the aqueous sol. The X ray diagrams obtained when the beam is perpendicular and parallel to the film, respectively, are shown in Figs 1*a, b*. They are typical wide angle α -patterns of the keratin myosin-epidermis fibrinogen group. The fact that Fig 1*b* is such a good approximation to a true fibre diagram indicates that the tropomyosin units are so elongated (and probably stiff) that the drying contraction normal to the surface of the sol forces them to lie down almost flat. The observed spacing of the characteristic meridian arc is 5.11 Å, but for the present there is some uncertainty about the side chain spacing. It is in any case always difficult to measure exactly, but in addition we have noticed an apparent variability at room humidities that, pending further investigation, might reasonably be ascribed to inter-chain swelling by water. The higher estimated values are of the order of 10 Å, which is rather greater than what is found for keratin and myosin. The photographs illustrated in Figs 1*a* and 2*a*—they were of an earlier preparation—show also an 'extraneous' halo at about 4.2 Å, which is a spacing that we have come to associate with traces of fatty substances, though it does not follow, of course, that that is necessarily the correct interpretation here.

It has not yet been found possible to produce β -tropomyosin by stretching the α film, either air dry or over water vapour, for it always breaks after a small percentage extension. In this respect it differs sharply from keratin and myosin. Neither has

squeezing at ordinary temperatures been found effective, though it succeeds with keratin, myosin, and fibrinogen (Astbury & Sisson, 1935, Astbury & Dickinson, 1940, Bailey, Astbury & Rudall, 1943). It appears for the moment that heat is necessary for instance, the disoriented but exceptionally well developed β diagram shown in Fig 2*a* was obtained by drying a moist specimen at 105°, while Fig 2*b* was obtained by squeezing another moist specimen between pieces of plate glass that had first been heated in steam, and then photographing with the X ray beam parallel to the flat surface. The transformation to β tropomyosin can take place at much lower temperatures, however, for a good β diagram was also obtained when the plates were preheated to only 80°.

Fig 2*b* is of the less familiar 'cross β ' type, i.e. with the backbone reflexion lying on the meridian instead of on the equator, and detailed examination shows that this is due to the fact that hot squeezing not only transforms the folded α -form into the extended β form, but also rotates the polypeptide grids so that their side chains stand approximately normal to the plane of flattening. This effect (it indicates a building up of ribbon like aggregates of β grids held together by the backbone linkage) was first observed on squeezing keratin in steam (Astbury & Sisson, 1935), and it was later observed on squeezing moist myosin even at room temperature (Astbury & Dickinson, 1940).

The backbone spacing is again 4.65 Å, as found in the other members of the *k m e f* group, but for the present the other two principal spacings, the side chain spacing and the amino acid spacing, are not so definite. As in the α diagram, the former appears to be somewhat higher than usual (it is probably over 10 Å at room humidity), while, in the absence of the normal β diagram, the latter can only be estimated at 3.2–3.3 Å.

Preliminary X ray examination has been made of tropomyosin crystals in the 'powder' form, air dry and moist, but nothing has been revealed so far beyond the disoriented α pattern, plus ammonium sulphate reflexions in the first case and a water halo in the second. It is clear that the α pattern arises from the structure of the individual tropomyosin units, if the crystals are redissolved and a film is made, the α pattern is found as before.

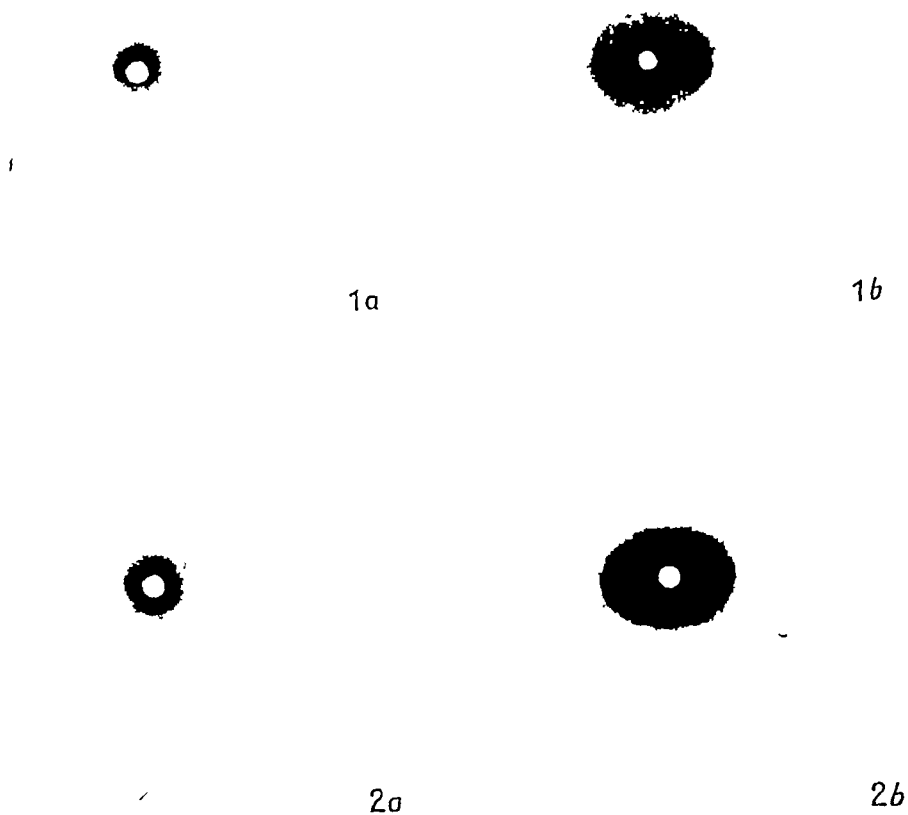


Fig 1 X ray photograph of tropomyosin film at ordinary humidity (a) Beam perpendicular to the surface (Disoriented α) (b) Beam parallel to the surface (Partially oriented α)

Fig 2 (a) Disoriented β diagram obtained by drying moist tropomyosin at 105° (b) Cross β diagram obtained by squeezing moist tropomyosin between glass plates that had been heated in steam Beam parallel to the surface

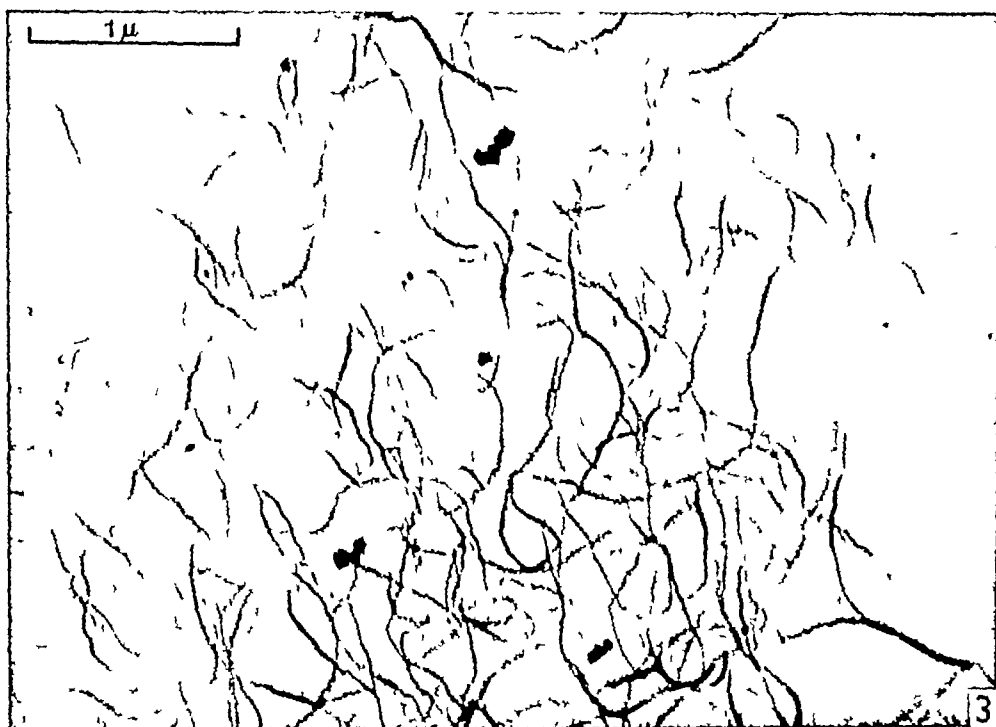


Fig 3 Tropomyosin fibrils deposited from aqueous solution

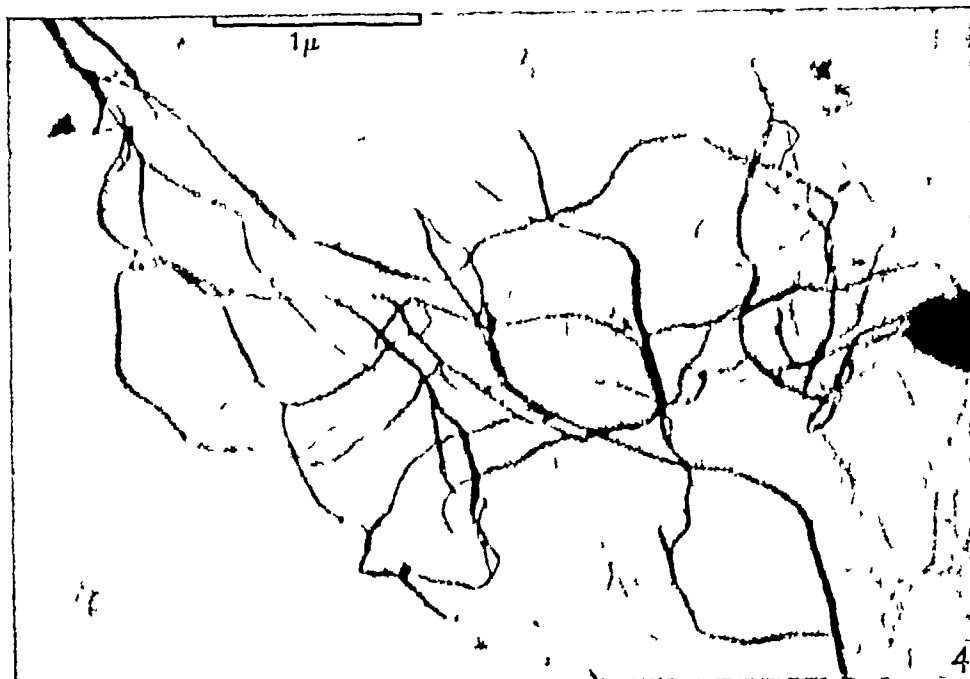


Fig 4 Tropomyosin fibrils deposited from aqueous solution and stained with osmic acid vapour



Fig 5 Tropomyosin fibrils deposited from aqueous solution and shadowed with gold

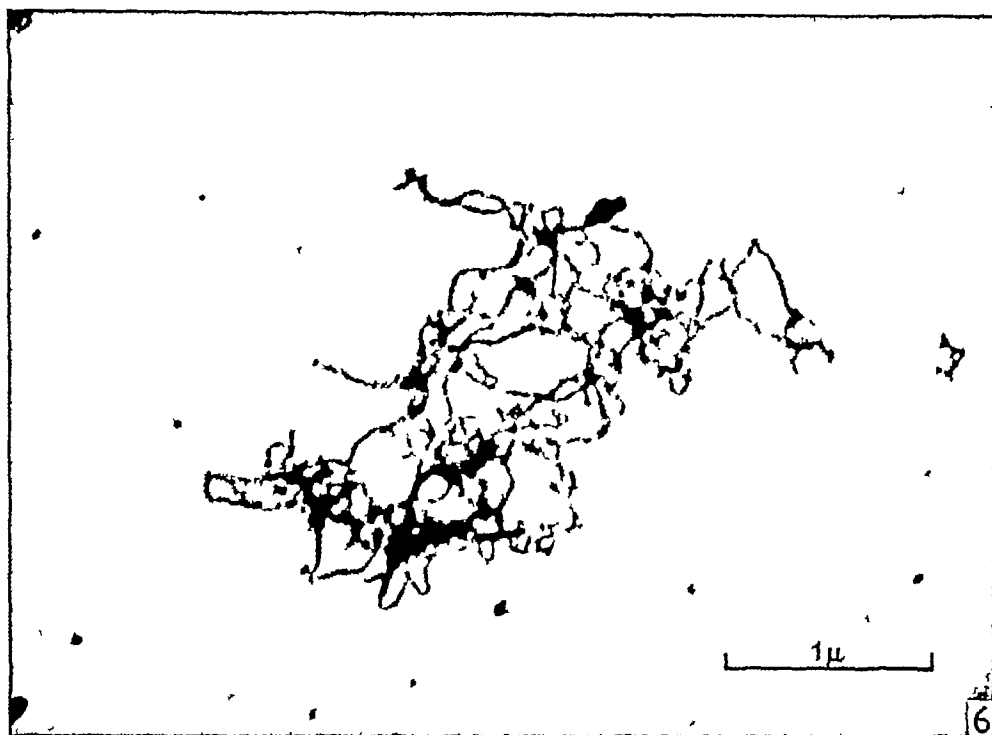


Fig 6 Tropomyosin fibrils deposited from an aqueous solution that had been heated to 80° for 5 min

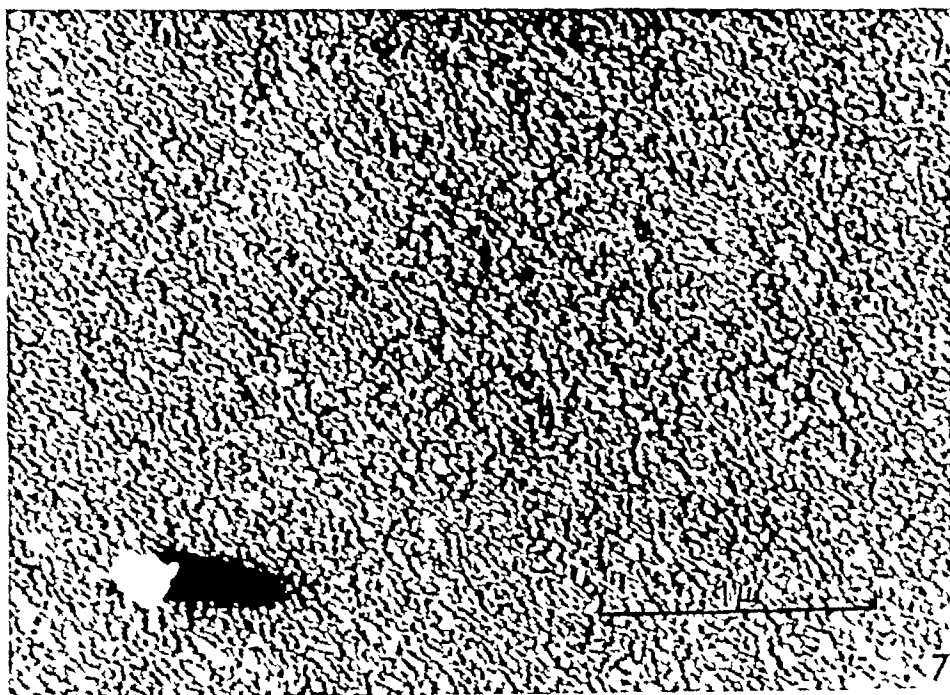


Fig 7 Deposit from a solution of tropomyosin in 0.1 M KCl Gold shadowed

A few preliminary observations have also been made on the property of 'supercontraction' that tropomyosin might be expected to possess in common with the other members of the *k m c f* group. It was found to be no exception. For this test, short lengths (about 1 cm) of narrow ribbon cut from a thin film were exposed to steam, and in every case a rapid contraction was recorded, amounting in the mean to some 30% of the initial length. These were admittedly only rough trial experiments, but they leave no doubt that tropomyosin, too, has the power of supercontracting strongly. It may be recalled that similar experiments with myosin films in which the molecules lay approximately flat, but in all azimuths, gave supercontractions of the order of 20% of the initial length (Astbury & Dickinson, 1940).

Electron microscope

The photographs were taken with an RCA instrument type B. The preparations studied were formed from drops of solution placed on filmed specimen grids and dried by one or other of the following means: air drying at room temperature, accelerated air drying at about 50°, desiccator drying, rapid vacuum drying. The aqueous solutions at pH 6-7, were made by dissolving in thrice filtered distilled water the horny solid obtained on drying a concentrated solution, and then diluting to about 1 in 50,000. Two salt solutions were examined, one in 0.1 M KCl and the other in 0.033 M KCl, at a concentration of 0.002% protein. For these a drop of solution was placed on the filmed grid and then immediately drained off with filter paper so as to leave a thin layer. The results described here were obtained with tropomyosin prepared from rabbit skeletal muscle but towards the end of the investigation similar photographs were obtained with fish tropomyosin.

Aqueous solutions were thus shown to give a mesh of very long, fine, and remarkably uniform fibrils, as illustrated in Fig. 3, which is a straightforward micrograph without either staining or metal shadowing.

Similar dry preparations deposited from aqueous solution were also exposed to the vapour of 1% osmic acid solution, at room temperature, for periods of 1-30 min., and afterwards washed with distilled water. This treatment made the fibrils much bolder in the viewing screen, and in addition it revealed in places a transverse banded, and somewhat saw-tooth, appearance. Fig. 4 illustrates a micrograph of this sort.

The most striking photographs (e.g. Fig. 5) were obtained after shadowing the dry preparations with gold, by the now well known technique developed by Williams & Wyckoff. The fibrils are seen to be of the order of 200-300 Å thick, and though there were sometimes indications of the banded or beaded appearance suggested by the osmic acid photographs, it was not found possible to confirm this finding outright because of suspicions that the gold

may have aggregated. However, it is hoped to repeat these experiments later using chromium.

The action of heat on aqueous solutions of tropomyosin is illustrated by Fig. 6. The solution was heated at 80° for 5 min. and a small drop was quickly transferred to a filmed specimen grid that had been warmed to 50° in a dry tube, after which the preparation was dried as quickly as possible. It will be seen that there is a well marked change in the character of the fibrils: the network has shrunk and the fibrils have less sharp outlines and have become curled up and often twisted together. Similar experiments at 100° or for longer times produced still more marked effects of this kind.

The action of KCl is illustrated in Fig. 7. The effect is to disperse the fibrils more or less completely, in fact, the 0.1 M solution gave hardly anything on which to focus. After staining with osmic acid vapour a few small fibrils and particles were revealed in the deposit from the 0.033 M solution, but from the 0.1 M solution there were fewer such particles and only poorly resolved micro fibrils, perhaps about 100 Å thick and a few hundred Å long.

DISCUSSION

The following discussion is based partly on the X-ray and electron microscope results just described and partly on data given in the preceding papers by Bailey (1948) and Bailey, Gutfreund & Ogston (1948).

Tropomyosin is distinguished from other members of the *k m c f* group (except perhaps fibrinogen) in that it consists of relatively short identical chains, or combinations of chains, from which can be prepared both fibres and visible orthodox crystals. It is a 'monomer' of the group (Astbury, 1947a, b), so to speak, the other members having proceeded presumably to the further stage of end to end addition to form much longer chains. It may be a breakdown product of some larger complex already built up in this way, or it may represent 'monomeric' units isolated before 'polymerization', but in either case there can be no doubt of its great importance for the study of the fibrous proteins. It is a key discovery that properly exploited might very well make the decisive contribution towards elucidating the *k m c f* group completely.

Taking the probable value of the molecular weight to be about 90,000, this is equivalent to 90,000/116.4 = 773 residues, and if these residues were incorporated into a single polypeptide chain in the fully extended configuration, their total length would amount to 773 × 3.33 = 2574 Å, and the average side dimensions of the chain would be approximately 10 Å by 4.5 Å. The mean axial ratio would thus be well over 300. We have, of course, to consider all possibilities in relation not only to X-ray

data but also to those provided by other techniques, but there is no really convincing argument in favour of this interpretation. Such diffusion and viscosity evidence as is available for the present, though admittedly imperfect, at least points to a considerably smaller axial ratio, and it is hard to believe that such long, flexible chains would build large crystals of the commonplace habit observed. The X-ray photograph given by tropomyosin in its normal state is always an α diagram, and quite apart from any question of the true nature of the α configuration, no doubt now remains as to what kind of diagram is given by the fully extended or β configuration: there is always a strong backbone reflexion of spacing 4.6 Å, not to mention other characteristic reflexions of weaker intensity. There is nothing in the normal tropomyosin diffraction pattern to suggest either extended chains of the full length quoted above or combinations of similar shorter chains: the diffraction pattern of aggregates of extended chains appears only after treatment to that end.

According to views most favoured at the moment (Astbury & Bell, 1941, Astbury, 1942), the α form is produced by a regular folding of the backbone (in a plane transverse to the side chains) which reduces the length by very nearly one half. On this interpretation we have, therefore, to consider chains of length about 1287 Å, or combinations of sub-multiple chains which are either distinct or are only loops in the longer chain. Table 1 gives the approximate dimensional characteristics of some of the more

Table 1 *Some possible dimensions of the tropomyosin molecule*

Effective no of α chains	Length (Å)	Side dimensions (Å.)	Mean axial ratio
1	1287	10×9.5	132
2	644	20×9.5 or 10×19	44
3	429	30×9.5 or 10×28.5	22

plausible possibilities. These dimensions refer, of course, to the anhydrous molecule, and they are intended to be no more than rough guides for comparison with results that may be obtained by other methods. The alternative side dimensions correspond to the two principal modes of linking parallel polypeptide chains, either by their side chains to form a 'grid' of two or three chains, or by their backbones, in which case they lie on top of one another like centipedes. The first possibility seems the more reasonable, but arguments can be adduced in support of the second.

Table 1, tentative as it is, serves at least to bring out the marked dimensional differences to be expected, and it should not be too difficult to distin-

guish between the various possibilities by means of critical measurements of diffusion, viscosity, and light scattering. Such measurements are for the present incomplete, but it is informative even now

Table 2 *Calculation of D and ν from possible axial ratios of the tropomyosin molecule*

(See text for explanation of columns)

(1) Mean axial ratio	(2) D_0/D	(3) D	(4) ν
132	~ 4.7	$\sim 1.54 \times 10^{-7}$	~ 996
44	2.78	2.60×10^{-7}	143
22	2.07	3.49×10^{-7}	45

to make a few comparisons. Table 2 gives estimates of quantities related to diffusion and viscosity. The explanation of the four columns is as follows:

(1) These axial ratios are taken from Table 1. For the purpose of subsequent calculations the assumption is that the molecule approximates to an unhydrated elongated ellipsoid.

(2) D_0 is the diffusion constant of a sphere of the same mass and volume as the ellipsoid. Its radius is given by

$$4/3\pi r^3 \times 1.28 = 90,000 \times 1.65 \times 10^{-24},$$

whence

$$r = 30.3 \text{ Å}$$

Also

$$D_0 = \frac{lT}{6\pi\eta r},$$

where l is Boltzmann's constant (1.380×10^{-16} erg/degree), T is absolute temperature, and η is the viscosity of the solvent (in this case a buffer at pH 6.5, consisting of $0.2M$ -KCl, $0.0133M$ Na_2HPO_4 , $0.0267M$ NaH_2PO_4 , for which Bailey found a viscosity of approximately 0.0098 poise). Thus D_0 at 20° is found to be 7.23×10^{-7} cm²/sec. The ratio D_0/D is equivalent to f/f_0 , the 'frictional ratio' of Svedberg & Pedersen (1940), and it is obtained from the axial ratio by aid of a table (Cohn & Edsall, 1943), based on F. Perrin's (1936) equation:

(3) D follows from the ratio D_0/D and the calculated value of D_0 .

(4) The quantity ν is the so-called 'viscosity increment' of the solute, and is given by

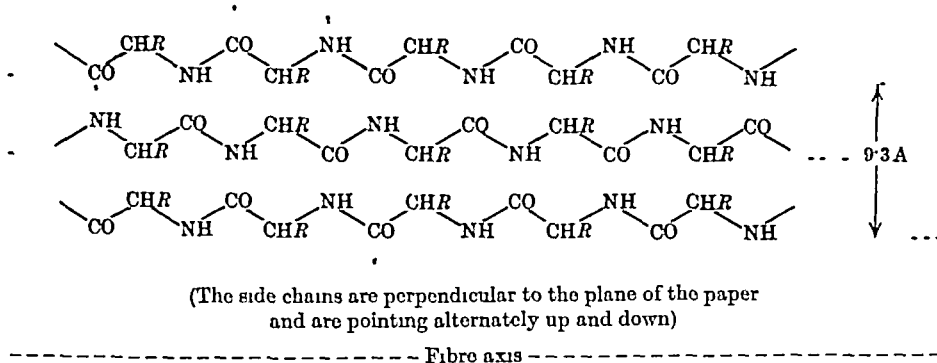
$$\frac{1}{\phi}(\eta/\eta_0 - 1) \equiv \nu,$$

where η is the viscosity of the solution, η_0 that of the solvent, and ϕ is the volume fraction of the system occupied by the solute molecules. It is obtained from the axial ratio by aid of a table based on Simha's equation (Mehl, Oncley & Simha, 1940; Simha, 1940; Cohn & Edsall, 1943).

The available experimental values of D are 2.22 and 2.26×10^{-7} found by Ogston, and 2.43×10^{-7} found by Gutfreund on extrapolating to a protein concentration of 0.6% the values obtained at concentrations of 1.2 and 0.7% . Bailey (personal communication) has found values of ν equal to 79 and 65 at ionic concentrations of 0.27 and 1.07 , respectively. If we assume that axial ratios intermediate between those quoted are ruled out—an

assumption which seems reasonable in the light of all that we know now about the *l m c f* group—then it is fair to say that the diffusion data support tolerably well the double α chain structure of mean axial ratio about 44. We should expect the observed diffusion constant to be appreciably less than the calculated, principally because we have made no allowance for hydration, which can hardly be neglected with such a polar molecule as tropomyosin, and because, strictly speaking, we need to know the diffusion constant either at infinite dilution or at least for a region of concentration where it does actually remain constant. These omissions represent only the more obvious difficulties there are, besides, the

The suggestion that a fundamental unit of the *l m c f* group comprises at least two parallel chains (either distinct or forming a loop in a single longer chain) is the more readily acceptable because there are also certain X ray indications to that effect (Astbury & Woods, 1933). The crystallographic interpretation of the β diagram requires a unit of pattern which repeats in one of the lateral directions at a distance of *twice* the backbone spacing, and our original explanation of this, which appears to be generally approved, was that it arises from two chains running lengthways but in opposite directions and linked by a succession of CO—NH bridges (now known as the 'backbone linkage') thus.



experimental difficulties associated with the exact measurement of diffusion constants, and in any case the theoretical formulae used are still to be considered as no more than hopeful approximations, pending thoroughgoing test on suitable molecules whose characteristics have been definitely established by independent methods. There would be little justification at this stage in trying to estimate, for example, the degree of hydration required to bridge the gap between prediction and observation, but on the whole it does appear that agreement with a double α chain is not unsatisfactory. As regards the viscosity data, however, the situation is less encouraging, for the present available values of ν point to something between the axial ratios of 44 and 22, that is, the observed viscosity is *less* than might be expected for a double α -chain. The explanation of this discrepancy may lie in Simha's formula, which is meant to apply only if orientation effects produced by the shearing forces are so small that all molecular orientations may be taken as equally probable. With greatly elongated structures such as we have reason to believe tropomyosin molecules are, the velocity gradients required to meet this condition must be very small indeed, otherwise the recorded viscosity will always be low. At present the experimental data are insufficient to guarantee that Simha's equation would be valid.

The electron microscope studies show that the very high viscosity and the easy flow birefringence of aqueous solutions of tropomyosin are consequences of the units aggregating into long, fine fibrils. The latter are observed visually only after evaporation, but there is no doubt that they exist as such in solution so long as salt is absent: when potassium chloride is added the fibrils are broken down again into their units, but the change is reversible on removing the salt. As already mentioned, it is remarkable how uniform the fibrils often are, as though they tend to an almost crystallographic regularity, but this is perhaps not surprising in view of the well formed and comparatively large crystals that can be grown by other procedures. The micrographs taken after treatment with osmic acid strengthen this impression by their occasional banded appearance, and it may be that some of the micrographs taken after gold shadowing, though at present open to suspicion, are to be interpreted similarly. By analogy with the yarn like structure of so many other fibres it would be natural to assume that the long tropomyosin units lie lengthways in the fibrils, overlapping one another irregularly in many regions, but lying strictly abreast or at least overlapping with geometrical regularity in those places where transverse bands are found, but it is not yet possible to verify this assumption directly.

by means of the X ray diagrams because of the difficulty of stretching strips of film without rupture. All that photographs of unstretched film show is that elongated units lie approximately parallel to the surface of the film, but whether parallel or transverse to the fibrils is uncertain, but if a suitable stretching technique could be devised, then it might be possible to orient the fibrils approximately parallel to a single direction and thus reveal which way the units lie *within* the fibrils. It seems very probable that they lie lengthways, otherwise we might expect the flow birefringence to be negative with respect to the direction of flow, whereas it is observed to be positive. Again, the observed usual fibril thickness is of the order of 200–300 Å (and finer fibrils are found too), which is less than the presumed length of even a three chain unit, let alone the two chain unit suggested by the diffusion data.

Tropomyosin fibrils present an important example of a phenomenon which has come to the fore only recently, but which promises at last to throw light on the mechanism of formation of protein fibres *in vivo*. It is now well established (Astbury, Dickinson & Bailey, 1935), and indeed has led to successful industrial development (cf. Astbury, 1945), that artificial protein fibres can be made by unfolding and subsequently drawing out the polypeptide chains from the specific configurations of many corpuscular proteins, but whether this process bears any relation to what happens in nature is another question. Actually, the evidence is beginning to point in a direction that is rather an extension of the familiar mechanism of chain formation by polymerization and polycondensation that has been exploited so strikingly in the manufacture of the other great group of man-made fibres, such as nylon and the like, which are truly synthetic. It is for this reason that we have considered tropomyosin as a kind of 'monomer' of the *l m e f* group (Astbury, 1947*a, b*): it has the capacity of building up fibres, but as so far observed *in vitro* these are only of an imperfect permanent character, the constituent units are held together by no more than secondary linkages and they can be dispersed again simply by the addition of salt. We can, however, conceive of a further and more permanent step whereby long fibres are built up by the incorporation of covalent linkages between the 'monomeric' units, and it is a 'polymerization' step of this kind that now seems one of the likely final stages in the formation of keratin, myosin and fibrin, and other natural protein fibres. Two other recently recognized examples of the reversible aggregation of corpuscular units to form long, uniform fibrils are fibrous insulin (Waugh, 1946, Hall, 1947) and F actin (Jakus & Hall, 1947, Astbury, Perry, Reed & Spark, 1947). The latter, discovered by Straub (1942, 1943), has been shown by Szent-Györgyi (1942, 1947) and his school to play a critical part in the mechanism of muscular contraction, but its significance in the deeper family relationships of the *l m e f* group may turn out to be more fundamental still (Astbury, 1947*b, c*).

The macroscopic crystals of tropomyosin that Bailey has succeeded in growing arouse the greatest interest. Above all they offer an opportunity of single crystal study by X-ray methods, with the

object of discovering ultimately at least the detailed shape if not the actual structure of the units. For the present what has been ascertained is that in the powder form the crystals give the same type of large angle diffraction pattern as is given by films and by the other members of the *l m e f* group, but it is hoped soon to be able to operate with single crystals. The thoroughly orthodox habit alone is something of a surprise, for it is the sort of thing one associates not with greatly elongated rods but rather with molecules much nearer the spherical, but even more astonishing is the very high water content, amounting to almost 90%. Bailey's figures are water 89.6, ammonium sulphate 1.74, and protein 8.76%, from which it follows that there are on the average about sixty-six water molecules per amino acid residue. Another way of describing the situation is to say that on the basis of parallel two chain units of lateral dimensions approximately 20×10 Å, each encased in a uniform thickness of water molecules, the mean distance between the surface of one unit and the next comes to about 40 Å. Of course, the longer side chains will project beyond the average, but in the case of the longest (arginine) this will only reduce the distance between the ends of side chains by perhaps 10 Å, and we are still left with the impression of a structure that is hardly more than an organized gel, an impression that is confirmed by the great difficulty in even moving the crystals without distorting them.

SUMMARY

1 The large angle X ray diffraction diagram of tropomyosin is the characteristic α diagram of the keratin myosin epidermis fibrinogen group. A similar diagram is obtained whether the specimen is thin film prepared from aqueous solution or a crystal 'powder' presumably, therefore, it arises from the intramolecular pattern of the tropomyosin units themselves.

2 It has not yet been found possible to produce the β diagram by stretching or squeezing at room temperature, but a well defined β diagram appears on heating to above about 80°, and hot squeezing orients the side chains perpendicular to the plane of flattening, as in other members of the *l m e f* group.

3 Preliminary tests indicate that tropomyosin film shows the property of 'supercontraction' found in other members of the *l m e f* group.

4 In the electron microscope, a deposit from aqueous solution is seen to consist of remarkably uniform fibrils mostly about 200–300 Å thick. These fibrils are completely dispersed into their constituent units by the action of potassium chloride.

5 When stained with osmic acid, and possibly also on shadowing with gold atoms, the fibrils show indications of cross striations.

6 Fibrils deposited from preheated aqueous solution have a shrunken, curled up and twisted appearance

7 The structures of the tropomyosin units, fibrils and crystals are discussed in the light of the above findings and data reported in the accompanying communications. It is suggested that tropomyosin is a typical 'monomer' of the *h m c f* group, the long fibres of which are built up by a process of 'polymerization' involving linkages of a more permanent character than those which operate in the formation of the fibrils found in aqueous solution

The latter, however, would appear to be of the nature of impermanent prototypes of the fibres formed *in vivo*

8 Available diffusion and viscosity data lend reasonable support to the hypothesis that the tropomyosin unit consists of a pair of chains, or a looped single chain, in the α configuration

It is clear that the investigation described in this paper could not have been carried out without Dr K. Bailey's close co-operation, and we wish gratefully to acknowledge this and the help of many discussions

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A Comparison of the Decomposition of Hydrogen Peroxide by Catalase, Ferrous and Ferric Ions, Haemin and Ferrous Phthalocyanine

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The catalytic decomposition of hydrogen peroxide into water and molecular oxygen has been shown to be a very complicated reaction. In the earlier work up to 1923 it was established that the reaction with many different catalysts is quasi unimolecular for instance, there are the typical experiments of Bredig & Berneck (1899), Duclaux (1922) and Bertalan (1920) on the decomposition catalyzed by colloidal metals and ferrous and ferric salts. From 1920 to 1932 more detailed kinetic analyses were carried out on the bromine bromide ion and iodine iodide ion

systems, and the first general kinetic mechanism for the decomposition was devised by Abel (1920) and Bray & Livingston (1923). This is the principle of compensating reactions in which both the oxidized and reduced forms of the catalyst react with the peroxide, giving a dynamic equilibrium between these two states and a resultant catalytic decomposition of the peroxide. For the heavy metal catalysis the chemical mechanism was represented by more or less cumbrous stoichiometric equations

The catalytic decomposition of the peroxide is a problem similar to that of the induced reactions studied extensively since the classical experiments of Manchot in 1901. In these reactions there are three components, *A* the actor, *B* the inductor and *C* the acceptor, all oxidizing or reducing agents. In the typical induced reaction *A* can react directly with *B* but not with *C*, also *B* does not react directly with *C* however, *C* does react with *A* if it is present with the reacting system *A* and *B*. The actor and inductor in many of the systems investigated have been oxygen or hydrogen peroxide and ferrous iron, and to explain the reactions higher oxides of iron, FeO_2 , Fe_2O_6 and FeO_3 , or iron peroxide complexes have been suggested (Hale, 1929).

In 1931 Haber & Willstätter suggested that free radicals are intermediates in many chemical reactions in solution, and in 1934 Haber & Weiss published a kinetic study of the decomposition by ferrous and ferric ions and proposed a chain mechanism for the reaction involving OH and O_2H radicals, which under certain experimental conditions could become a more simple sequence of radical reactions.

The principle of compensating reactions has been widely accepted for the catalysis of the bromine bromide ion type, and the Haber & Weiss mechanism has proved an extremely useful working hypothesis for the catalysis by metal ions. It has recently been developed very successfully by Baxendale, Evans & Park (1946) to account for the initiation of the polymerization of certain olefins by ferrous sulphate and hydrogen peroxide in acid solution.

In this and following papers a detailed kinetic analysis of the reaction with various catalysts will be described. There are three features of the decomposition as yet uncorrelated which have directed the experimental work. They derive in turn from the three lines of development of the subject outlined above.

(1) In many of the catalytic systems there is initially a rapid evolution of oxygen, followed by a much slower evolution. This is known as α - and β activity. It suggests that some steady state is reached in agreement with the principle of compensating reactions.

(2) During this transition from α - to β -activity the equivalent of peroxide destroyed may be in excess of the equivalents of catalyst present. Haber & Weiss (1934) found this for the decomposition with ferrous iron, and it is their chief evidence for the chain character of the reaction. Many oxidase reactions show the same behaviour. Stern has stressed this point in a recent review (1942). The same behaviour occurs in most of the induced reactions. The participation of higher oxides of iron or iron-peroxide complexes was put forward to explain the oxidation of two or three molecules of

acceptor whilst the ferrous iron is converted to ferric iron, and side reactions of the higher oxide or complex with hydrogen peroxide, regenerating ferrous iron, can account for greater turnover numbers.

(3) Leaving aside the question of the kinetic mechanism of the reaction, the accepted idea of single electron transfers in this type of oxidation-reduction system makes it necessary to assume the participation of free radical intermediates in the several unit reactions by which the overall decomposition of the peroxide occurs.

Experiments on the initial evolution of oxygen in the reaction between hydrogen peroxide and catalase, ferrous and ferric ions, haemin and ferrous phthalocyanine are described in this paper, and possible explanations for the transition from α to β -activity are critically examined.

METHOD

The rate of O_2 evolution has been investigated for reaction mixtures of H_2O_2 and the following catalysts: haemin, ferrous phthalocyanine, ferrous ammonium sulphate, ferric sulphate and liver catalase. The liver catalase was a highly purified preparation obtained from horse liver by the method described by Keilin & Hartree (1945). The kinetic measurements were carried out in two different types of apparatus, Barcroft manometers and a special pressure gauge apparatus constructed for following the very rapid evolution of O_2 which occurs in the initial stages of the reaction with certain catalysts. Tests were made to ensure that the rate of O_2 evolution measured was a true chemical rate and not conditioned by any physical factors such as diffusion.

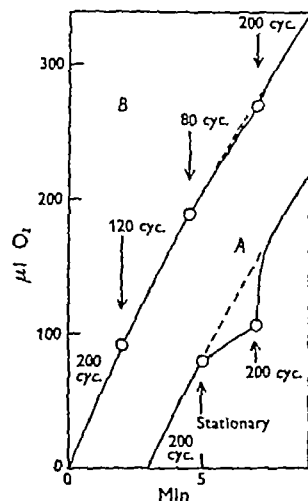


Fig 1 Diffusion test with Barcroft manometers. Temp 20° , pH 5.85, oxygen evolved from H_2O_2 + catalase. Shaking speed in cyc/min.

Diffusion test with Barcroft manometers. In these experiments the left-hand flask contained 3.30 ml H_2O and the right-hand flask 0.30 ml 0.125M phosphate buffer, pH 5.85, 0.10 ml 2% gelatin solution, 1.10 ml water and 1.70 ml H_2O_2 (about 20 vol) to bring the solution to 1.0M, together

with the dangling tube holding 0.10 ml. dilute catalase solution. The effect on the reaction rate of keeping the manometers stationary and of varying the shaking speed was determined. The results are given in Fig. 1 from curve A it is apparent that physical conditions limit the rate measured when the manometer is stationary, but that on recommencing rapid shaking the true chemical rate is once more established. The latter part of curve A is an accurate extrapolation of the former part before shaking was stopped and so the chemical reaction proceeds at the same rate whether the flask is shaken or not. Curve B gives similar experimental results on varying the speed of shaking. At shaking speeds above 80 cyc/min the true chemical rate can be measured—a speed of 150 cyc/min was used throughout this work.

Diffusion tests with the pressure-gauge apparatus. This apparatus is a simple modification of that described by Meldrum & Roughton (1934) as the 'boat technique'. In

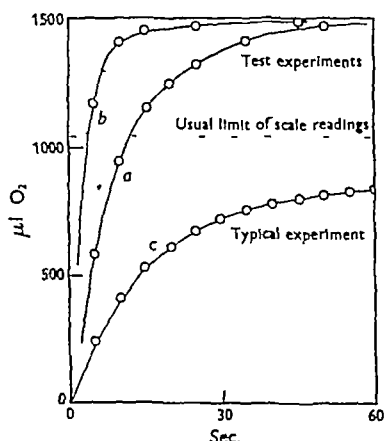


Fig. 2 Diffusion test with pressure gauge apparatus. Temp. 0° , a and b, test experiments with $\text{H}_2\text{O}_2 + \text{KMnO}_4$, c, typical experiment with $\text{Fe}^{++} + \text{H}_2\text{O}_2$.

place of the oil manometer for measuring the evolution of gas a pressure gauge was used. This consists of a flat brass cylinder (12 cm diam., 2 cm deep) soldered at the bottom to a heavy brass plate. A thin brass diaphragm is fitted over the top and held in place by a screwed flange, which enables the tension on the diaphragm to be altered. A fine steel pin (1 cm. high) is fixed to the centre of the diaphragm; the pin is in contact with one end of a small duralumin lever, pivoted about its centre, which carries a galvanometer mirror at the other end. By means of a galvanometer lamp and scale the slight movements of the diaphragm caused by pressure changes in the apparatus are very much magnified. The apparatus is very sensitive to rapid changes of pressure, the lag time is much less than 0.1 sec. The tension on the diaphragm was adjusted, and a test with a subsidiary oil manometer in the system showed that over the range 0–120 scale divisions the deflexion was directly proportional to the pressure change (1 scale division $\equiv 0.336$ mm. paraffin). The scale can be read to half a division. A set of six boats was calibrated for the apparatus by both the chemical method (acidified KMnO_4 and H_2O_2) and the direct method described by Münzer & Neumann (1917). One scale division is approximately equal to $9.2 \mu\text{l}$ of O_2 . In experiments with this apparatus the scale reading was recorded every 5 sec.

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Diffusion tests were carried out at 0° with the same solutions employed in the chemical calibration. The two sides of the boat were filled:

- (i) 1.5 ml. 0.0875N H_2O_2 + 0.5 ml. H_2O
- (ii) 2.0 ml. 0.195N KMnO_4 in 0.5N H_2SO_4 .

The evolution of O_2 from this mixture (1480 μl) is given in Fig. 2. Curves a and b show the effect of medium and very fast shaking. The points on curve b were independent of any further increase in the shaking speed, so this curve actually represents the chemical rate of the KMnO_4 – H_2O_2 reaction. The apparatus is clearly capable of measuring true chemical rates of at least 200 $\mu\text{l}/\text{sec}$. The very fast shaking speed was always employed. The results of a typical experiment are shown in curve c for the evolution of O_2 from acidified ferrous ammonium sulphate and H_2O_2 . The experimental details are:

Boat filled (i) 0.5 ml. $\text{FeSO}_4(\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$ solution (12 g./100 ml.) and 1.5 ml. very dilute H_2SO_4
(ii) 0.37 ml. H_2O_2 + 1.63 ml. water

Final concentrations: 0.2M H_2O_2 , acid $\equiv 0.142$ M H_2SO_4 .

RESULTS

With some catalysts there is a very rapid evolution of O_2 at the beginning of the reaction which quickly subsides leaving the decomposition proceeding at a much slower rate. The experiments that follow are designed to show whether this effect is to be attributed to the chemical destruction of the catalyst, or whether it is a general kinetic feature of the decomposition arising from the interplay of chemical reactions.

In all the experiments the total volume of O_2 evolved is very small compared with the H_2O_2 concentration present, and so it can be assumed for the kinetic analysis that the peroxide concentration remains constant throughout each experiment. Control experiments with no catalyst present showed the 'blank' reaction to be negligible.

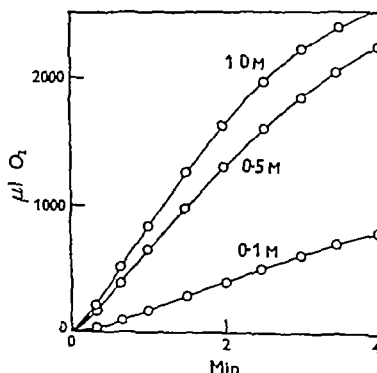


Fig. 3 Haemin. Oxygen evolution with time. Temp. 0° , pH 5.85, identical haemin concentrations. H_2O_2 1.0, 0.5 and 0.1 M.

Oxygen evolved with haemin. Since haemin is very rapidly destroyed by H_2O_2 at room temperature, these experiments were carried out at 0° with the

pressure gauge apparatus The O_2 evolved during the first 4 min at three different H_2O_2 concentrations is shown in Fig 3

The boats were filled

(i) 0.5 ml haemin solution (0.117 g dissolved in a little NaOH and made up to 50 ml), 0.5 ml 0.125M phosphate buffer, pH 5.85, + 1.0 ml water

(ii) 2 ml H_2O_2 + H_2O to give required final molarity

Oxygen evolved with ferrous phthalocyanine These experiments were carried out in Barcroft manometers at 20°

The flasks were filled

Left-hand side, 3.30 ml H_2O

Right-hand side, 0.5 ml 0.125M phosphate buffer, pH 5.85 H_2O_2 + water to 3.2 ml, giving required molarity
Dangling tube 0.1 ml iron phthalocyanine suspension containing 0.2 mg dry weight/ml

Fig 4 shows the O_2 evolution at three H_2O_2 concentrations during the first 5 min

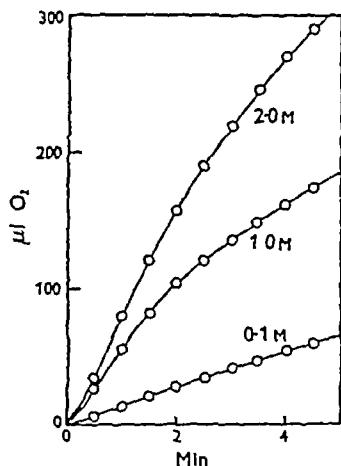


Fig 4 Ferrous phthalocyanine Oxygen evolution with time Temp 20°, pH 5.85, identical ferrous phthalocyanine concentrations H_2O_2 2.0, 1.0 and 0.1M

Oxygen evolved with ferrous iron These experiments were also carried out in Barcroft manometers at 20°. No buffer solution was used 0.1 ml of a solution of acidified $FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$ (1.66 g / 7 ml) was placed in the dangling tube, the total acid concentration present being equivalent to 0.565 ml N H_2SO_4 . The results for 1.0M and 0.05M H_2O_2 are given in Fig 5

Oxygen evolved with ferric iron The decomposition of H_2O_2 in the presence of ferric salts falls off very rapidly as the acidity of the solution is increased. Ferric sulphate in aqueous solution, owing to its hydrolysis, has a very acid reaction, and to obtain a measurable O_2 evolution in these experiments it was necessary to add a very small quantity of NaOH. The amount added was not enough to alter

the state of the ferric iron the solution still had the pale yellow colour characteristic of the ferric iron in acid solution. The experiments were carried out in the pressure gauge apparatus at 0°. 1.0 ml of a solution of $Fe_2(SO_4)_3 \cdot 9H_2O$ (8.7 g / 50 ml) + NaOH equivalent to 0.03 ml N-solution was used in each run, together with H_2O_2 and H_2O to give the required molarity. Typical results are shown in Fig 6

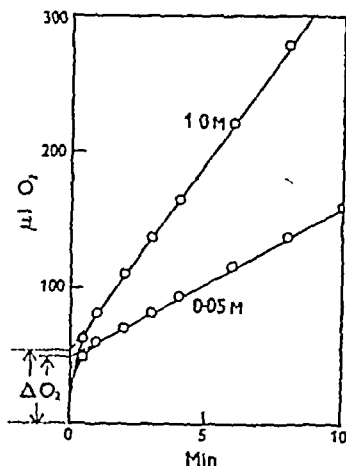


Fig 5 Ferrous iron Oxygen evolution with time, showing initial activity Temp 20°, acid solution, identical Fe^{++} concentrations H_2O_2 1.0 and 0.05M

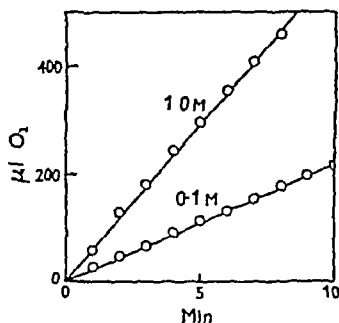


Fig 6 Ferric iron Oxygen evolution with time Temp 0°, acid solution, identical Fe^{+++} concentrations H_2O_2 1.0 and 0.1M

Oxygen evolved with liver catalase Barcroft manometers were used at 20°

Into the right-hand flask were measured 0.3 ml 0.125M phosphate buffer, pH 5.85, + 0.1 ml 2% gelatin solution together with H_2O_2 and water for the required molarity. A very dilute catalase solution (0.1 ml) was put in the dangling tube. This solution was prepared as follows: stock solution A contained 34 mg catalase/ml. One drop of this solution (0.045 g) was added to 20 ml water giving solution B. Solution B (0.6 ml) was made up to 50 ml. This was kept in ice in a thermos flask, 0.1 ml being used for each run.

Typical results for the decomposition with 1.0M and 0.2M H_2O_2 for the first 15 min are given in Fig 7. Fig 8 shows the results of a similar experiment lasting for 1 hr. Here the catalase concentration was one sixth of its previous strength.

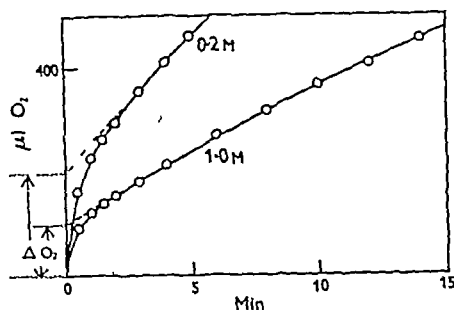


Fig 7 Liver catalase. Oxygen evolution with time showing initial activity. Temp 20° , pH 5.85, identical catalase concentrations. H_2O_2 1.0 and 0.2M.

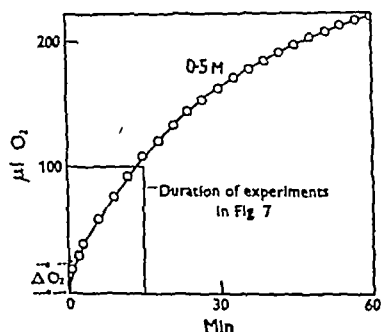


Fig 8 Catalase. Oxygen evolution with time showing a diminished activity after 15 min, attributed to destruction of the enzyme. Temp 20° , pH 5.85, 0.5M H_2O_2 .

DISCUSSION

The destruction of the catalyst

An examination of the experimental curves for the various iron containing catalysts studied shows that kinetically the catalysts are of two types. Ferrous iron and catalase bring about a reaction in which there is an initial rapid evolution of O_2 which is followed by a much slower steady decomposition. Haemin, ferrous phthalocyanine and ferric iron do not give this initial 'burst' but only the steady rate. The initial evolution in the catalase reaction is extremely interesting; it has been observed by other investigators (Morgulis, Beber & Rabkin, 1926), but as yet no attempt has been made to explain it kinetically and account for it in the mechanism proposed for the reaction. It might be due to the destruction of the enzyme or to some special feature in the decomposition of the peroxide. The observed

behaviour with the other catalysts strongly supports the second explanation for the following reasons:

(1) The reaction with catalase and with ferrous iron is very similar (Figs 5 and 7). With ferrous iron

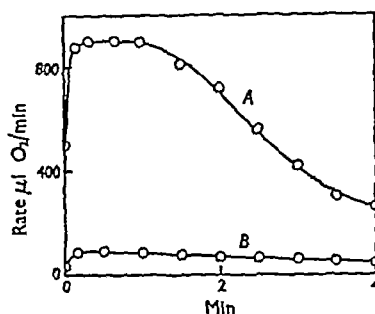


Fig 9 Variation of rate with time. Values plotted from Figs 3 and 4. A, haemin + 1.0M H_2O_2 ; B, ferrous phthalocyanine + 2.0M H_2O_2 .

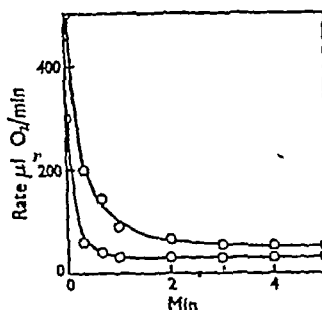


Fig 10 Variation of rate with time. Values plotted from Figs 5 and 7. Bottom curve ferrous iron + 1.0M H_2O_2 . Top curve catalase + 0.2M H_2O_2 .

no destruction of the catalyst can occur, and the kinetic effect here is due to the oxidation of ferrous to ferric iron by the peroxide (Haber & Weiss, 1934, Baxendale *et al.* 1946). There is no initial 'burst' with ferric iron (Fig 6).

(2) With haemin and ferrous phthalocyanine destruction of the catalyst does occur. The solutions rapidly lose their characteristic colours. This is very marked at high peroxide concentrations (1.0M and 2.0M) when the rate falls off after the first minute. At low concentration (0.1M H_2O_2) this is hardly noticeable. The initial rapid evolution with catalase and ferrous iron is just as marked at low as at high concentrations. This behaviour can be demonstrated by plotting 'rate' against 'time' as in Figs 9 and 10; the shape of the curve is completely different in the two cases. With catalase and ferrous iron the rate rapidly falls to an almost constant value (Fig 10), whereas with haemin and ferrous phthalocyanine the rate continually decreases (Fig 9).

(3) After the initial activity is over the reaction with catalase proceeds at this almost steady rate, which above a certain peroxide concentration is less the higher the peroxide concentration (Fig. 7). This will be described in detail in a later paper. If enzyme destruction is responsible for this initial activity the rate should diminish continuously to zero. These constant rates at different peroxide concentrations cannot be explained by the enzyme destruction hypothesis.

(4) Enzyme destruction does occur with catalase, but it is only apparent after a much longer time. This is shown in Fig. 8: here the curve from 2 min. to 1 hr. resembles that for haemin from 0 to 5 min.

Thus, whilst the complete destruction of the enzyme cannot be responsible for these kinetic effects, there remains the further possibility that the initial activity results from its partial destruction, leaving a degradation product which is still extremely catalytically active. In a later paper, in which a detailed kinetic study of the reaction will be described, it will be shown that these results cannot be accounted for in this way, and so the initial activity is due to some special feature in the catalytic decomposition of the peroxide.

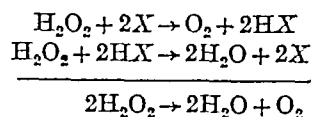
The following correlation can be made between the state of oxidation of the catalyst and its kinetic effect. The different kinetic behaviour of the various catalysts, the initial rapid evolution of O_2 observed with some and its absence with others, depends on the state of oxidation of the catalyst initially and the concentration of this state in the equilibrium mixture obtained in peroxide solution. In the ferrous/ferric iron system the equilibrium mixture contains predominantly ferric iron, and so if ferrous iron is added to H_2O_2 there is a rapid evolution of O_2 whilst the oxidation of Fe^{++} to Fe^{+++} proceeds. With ferric iron, haemin and ferrous phthalocyanine the initial oxidation state of the catalyst corresponds to that predominating in the equilibrium mixture, and so there is no initial rapid evolution. Catalase, although containing ferric iron, is in the same kinetic class as free ferrous iron. In H_2O_2 solution the initial rapid evolution of O_2 suggests that some type of oxidation-reduction equilibrium is being attained.

The principle of compensating reactions, which provides a qualitative explanation for this, will now be discussed and its quantitative aspects examined. It will be realized that this principle is the general kinetic mechanism for the simple valency change reactions (with the exception of the chain reaction) which have been proposed as mechanisms for the decomposition.

The principle of compensating reactions

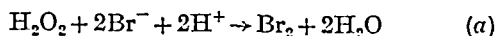
The simple picture is that of two reactions, in one the oxidized form of the catalyst is reduced by the peroxide liberating oxygen, in the other the reduced

form of the catalyst is oxidized by the peroxide giving water. The net effect of these compensating reactions is the familiar catalytic decomposition

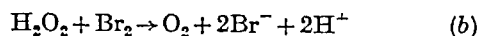


This mechanism has been reviewed by Bray (1932); it has been widely used to explain the catalysis by halogens and the corresponding halide ions. These correspond to the oxidized and reduced state of the catalyst. The mechanism may be illustrated by the decomposition by Br_2 and Br^- .

If H_2O_2 is added to a solution containing Br^- at high H^+ concentration, Br_2 is rapidly formed and soon reaches a constant concentration

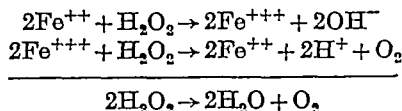


If H_2O_2 is added to bromine water when either H^+ or Br^- concentrations are low, Br_2 is rapidly reduced to Br^- and oxygen is evolved



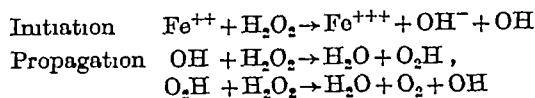
If the temperature and the final concentrations of H^+ and Br^- are the same as in (a), an identical bromine concentration is reached which again remains constant. Under these conditions the rates of the two reactions (a) and (b) are equal. H_2O_2 is decomposed catalytically—this is the steady state. O_2 is evolved in reaction (b), but not in (a), so if H_2O_2 is added to bromine water a transition from α to β activity will be observed. The reaction with certain heavy metal catalysts is very similar kinetically.

However, for the catalysis by ferrous and ferric ions this simple picture is not sufficient. The obvious compensating reactions in this case are



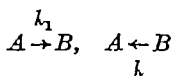
Now in the oxidation of Fe^{++} to Fe^{+++} by this mechanism, whilst there would be a rapid destruction of H_2O_2 in attaining the equilibrium state, yet there would be no rapid evolution of O_2 , since this arises in the reaction of ferric ion only. This shows conclusively that the reaction is more complicated. The experiments of Haber & Weiss (1934) led them to propose the chain mechanism which can account for it. They measured ΔH_2O_2 , the amount of peroxide destroyed in the oxidation of Fe^{++} to Fe^{+++} in neutral solution. Under certain experimental conditions they found that the ratio of ΔH_2O_2 to the initial equivalent concentration of Fe^{++} was greater than one, and attributed this to the length of a chain reaction, initiated by the production of OH radicals.

by Fe^{++} and H_2O_2 and propagated by the reactions of the OH and O_2H radicals



The oxygen produced in the second chain propagation reaction explains its evolution in the oxidation of Fe^{++} to Fe^{+++} which could not be accounted for by the simple compensating reactions outlined above. In the gasometric experiments described in this paper the corresponding ratio $\Delta\text{O}_2/E_0$, the amount of oxygen evolved in reaching the equilibrium state, to the molar concentration of catalyst initially present can be evaluated. This is important because if the ratio is less than one then the principle of compensating reactions provides an adequate kinetic mechanism; if it is greater than one then either a chain mechanism or another more complex reaction mechanism must be adopted.

In the appendix to this paper the mathematical expression for the ratio $\Delta\text{O}_2/E_0$ is derived from the general system of two compensating reactions. If A and B are the two oxidation states of the catalyst, it takes part in two opposing monomolecular reactions which can be represented



The parameters k_1 and k_2 contain peroxide and possibly H^+ ion concentrations together with the reaction velocity constants. When oxygen is evolved in the first reaction and not in the second it is shown that the ratio

$$\frac{\Delta\text{O}_2}{E_0} = \frac{k_1^2}{(k_1 + k_2)^2}, \quad \text{if } \frac{\Delta\text{O}_2}{E_0} < 1$$

If the compensating reactions are more complicated and oxygen is evolved in both steps then the ratio is still less than one. This ratio can be calculated for the experiments in which ferrous iron and catalase were used.

Ferrous iron (1.0 and 0.05M H_2O_2),

$$\Delta\text{O}_2 = 50 \mu\text{l} = 2.2 \times 10^{-6} \text{ mol}$$

$$\text{Fe}_2^{3+} = 6.1 \times 10^{-5} \text{ mol}$$

$$\text{Fe}_2^{3+}/\Delta\text{O}_2 = 0.037$$

Thus, with the experimental conditions employed, there is no evidence for a chain reaction and a set of compensating reactions is sufficient.

Catalase

$$E_0 = 4.1 \times 10^{-13} \text{ mol}$$

$$\Delta\text{O}_2 (1.0\text{M } \text{H}_2\text{O}_2) = 100 \mu\text{l}, \quad \frac{\Delta\text{O}_2}{E_0} = 1.1 \times 10^7$$

$$\Delta\text{O}_2 (0.2\text{M } \text{H}_2\text{O}_2) = 200 \mu\text{l}, \quad \frac{\Delta\text{O}_2}{E_0} = 2.2 \times 10^7$$

This is a most striking result, for the ratio is of the order ten million, rather than less than one. It is apparent that the system of two compensating reactions is not an adequate kinetic mechanism. The applicability of the chain mechanism or another more complex mechanism must be examined.

It can be argued from the experiments of Haber & Weiss (1934), on the decomposition by ferrous ions, that this result means that with catalase a chain reaction occurs with a chain length of ten million. However, a comparison of the results with catalase and with ferrous iron presents a serious difficulty. It follows from the mathematical treatment of the compensating reactions given in the appendix that the time for half the initial rapid evolution to occur, t_1 , is given by

$$t_1 = \frac{\ln 2}{(k_1 + k_2)}$$

So, from the experimental results, the sum of $k_1 + k_2$ can be evaluated. With 1.0M H_2O_2 the values are

$$\text{Catalase} \quad t_1 = 10 \text{ sec}, \quad (k_1 + k_2) = 0.069 \text{ sec}^{-1}$$

$$\text{Ferrous iron} \quad t_1 = 5 \text{ sec}, \quad (k_1 + k_2) = 0.139 \text{ sec}^{-1}$$

The sum $k_1 + k_2$ is of the same order for both ferrous iron and catalase. Now according to the Haber & Weiss (1934) mechanism, where the catalyst both starts and stops reaction chains k_1 and k_2 , the parameters involving velocity constants, H_2O_2 and possibly H^+ ion concentrations, refer to the chain starting and chain stopping reactions. The measurements of $\Delta\text{O}_2/E_0$ suggested that, whilst the ferrous iron reaction does not involve long chains (a sequence of reactions provides an adequate mechanism), the catalase reaction has a chain length of 1.1×10^7 with 1.0M H_2O_2 . The corresponding chain length for the Fe^{++} ion reaction, 0.037, can be interpreted on the Haber & Weiss mechanism as a more rapid removal of chain centres by reaction with the Fe^{++} ion in the termination process compared with the alternative reaction of the chain centres with H_2O_2 in the propagation reaction. On this basis the ratio of the velocity constants $(k_1^2)_{\text{Fe}}$ and $(k_1^2)_{\text{E}}$ for the termination reactions in the Fe^{++} ion and enzyme systems can be estimated. If k_p is the velocity constant for the propagation reaction, then

$$\frac{k_p [\text{H}_2\text{O}_2]}{(k_1^2)_{\text{Fe}} [\text{Fe}^{++}]} = 0.037 \quad \text{and} \quad \frac{k_p [\text{H}_2\text{O}_2]}{(k_1^2)_{\text{E}} [\text{E}]} = 1.1 \times 10^7$$

Substituting the experimental values for H_2O_2 , Fe^{++} and catalase concentrations, the ratio $(k_1^2)_{\text{Fe}}/(k_1^2)_{\text{E}}$ is found to be 2.0. This implies that the velocity constants of the chain initiation and termination reactions for catalase and ferrous iron are all of the same order. Whilst the H^+ ion concentration is considerably different in the two cases it can hardly account for this odd conclusion. In all comparable

reactions of the iron protoporphyrin protein complexes their velocity constants greatly exceed those with free iron. Thus the simple chain theory does not offer a satisfactory explanation for the action of catalase and the possibility of other explanations should be explored. One alternative mechanism was suggested by Manchot during the course of his work on induced reactions.

Manchot & Lehmann (1928) described experiments with ferrous iron and H_2O_2 , the results of which are very similar to those of Haber & Weiss (1934). In the oxidation of Fe^{++} to Fe^{+++} in dilute solution he found that, for each equivalent of Fe^{++} oxidized, three equivalents of peroxide were decomposed, two equivalents of which could react with acceptors. In more concentrated peroxide solutions as many as twenty-four equivalents of peroxide were decomposed for the oxidation of each equivalent of Fe^{++} . He explained the result in dilute solution by the intermediary formation of Fe_2O_6 , and in the more concentrated peroxide solutions the increased peroxide decomposition is satisfactorily explained by a reaction between the intermediate and H_2O_2 , in which the latter reduces the former back to the ferrous state.

In the absence of more kinetic data it is impossible to reach any definite conclusion regarding the mechanism of the decomposition. Detailed kinetic studies of the reaction with catalase and ferrous iron will be described in future papers.

SUMMARY

1 The evolution of O_2 from H_2O_2 by haemin, ferrous phthalocyanine, ferrous and ferric iron and catalase has been measured manometrically and by

the 'boat technique' of Meldrum & Roughton (1934), using a pressure gauge for following rapid rates.

2 There is an initial rapid evolution of O_2 with ferrous iron and catalase but not with the other catalysts. In general, this transition from α - to β activity might be due to the destructive fission of the catalyst, or to a special kinetic feature of the decomposition with a particular catalyst. Since ferrous iron shows this activity it is not necessarily due to catalyst destruction, for none can occur here.

3 By comparing the catalytic effect of catalase with haemin and iron phthalocyanine it has been shown that complete destruction of the enzyme cannot account for the initial activity, and it is unlikely that partial destruction is responsible.

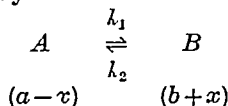
4 The principle of compensating reactions, which attributes the $\alpha\beta$ activity to the setting up of an equilibrium between two oxidation states of the catalyst, can explain the ferrous iron catalysis but not the reaction with catalase. This follows from the values of $\Delta\text{O}_2/E_0$, the ratio of the equivalents of oxygen evolved in the initial rapid reaction to the equivalents of catalyst present, which are 0.037 and 1.1×10^7 respectively for the given experimental conditions.

5 For the catalase reaction the familiar chain mechanism leads to the improbable conclusion that the reaction velocity constants for chain initiation and termination are each of the same order of magnitude with catalase and ferrous iron.

I am very grateful to Prof. D. Keilin, F.R.S., for his stimulating interest and kind encouragement in this work and I wish to thank Dr E. F. Hartree for much advice on manometric methods and the preparation of catalase.

Appendix Compensating Reactions and the Initial Evolution of Oxygen

The relation between the number of equivalents of oxygen evolved in the initial rapid reaction and the equivalents of catalyst present can be obtained in the following way.



A and B represent the two oxidation states of the catalyst whose initial concentrations and concentrations at time t are given by a and b , and $(a-x)$ and $(b+x)$ respectively. At time t the rates of conversion of A into B and B into A are given by $k_1(a-x)$ and $k_2(b+x)$, the parameters k_1 and k_2 containing peroxide and possibly hydrogen-ion concentration terms. This is the simplest representation of two compensating reactions, if either involves a sequence of reactions then k_1 and k_2 refer to the rate determining steps in such a sequence.

Moelwyn Hughes (1940) has shown that in such a system of two compensating monomolecular reactions (monomolecular with respect to the catalyst in the peroxide decomposition)

$$x = \frac{k_1 a - k_2 b}{k_1 + k_2} (1 - e^{-(k_1 + k_2)t})$$

Now if $b=0$, that is the catalyst is present initially in the ' A ' state, then the amount of A left at any time is $(a-x)$ where

$$(a-x) = a \frac{k_2 + k_1 e^{-(k_1 + k_2)t}}{k_1 + k_2}$$

Changing to a more familiar nomenclature, if E_0 is the initial concentration of the catalyst (in state A) and E is its concentration at time t ,

$$E = E_0 \frac{k_2 + k_1 e^{-(k_1 + k_2)t}}{k_1 + k_2}$$

Now if the k_1 reaction is the one in which oxygen is evolved, the rate at any time R_t is given by

$$R_t = k_1 E = \frac{E_0 k_1 [k_2 + k_1 e^{-\overline{k_1+k_2}t}]}{k_1 + k_2}, \quad (1)$$

and R_0 and R_e , the initial and equilibrium rates when $t=0$ and $t=\infty$ respectively, are

$$R_0 = k_1 E_0, \quad (2)$$

$$R_e = E_0 \frac{k_1 k_2}{k_1 + k_2} \quad (3)$$

The relation between the amount of oxygen evolved in reaching the equilibrium and the mol of catalyst present is obtained by integrating equation (1)

$$\left. \begin{aligned} R_t &= \frac{dO_2}{dt} = \frac{E_0 k_1 k_2}{k_1 + k_2} + \frac{E_0 k_1^2}{k_1 + k_2} e^{-\overline{k_1+k_2}t}, \\ dO_2 &= \frac{E_0 k_1 k_2 dt}{k_1 + k_2} + \frac{E_0 k_1^2}{k_1 + k_2} e^{-\overline{k_1+k_2}t} dt \end{aligned} \right\} \quad (4)$$

Integrating this expression

$$O_2 = \frac{E_0 k_1 k_2 t}{k_1 + k_2} - \frac{E_0 k_1^2}{(k_1 + k_2)^2} e^{-\overline{k_1+k_2}t} + I, \quad (5)$$

where I is the integration constant. This can be evaluated, for when $t=0$, $O_2=0$, therefore

$$I = \frac{E_0 k_1^2}{(k_1 + k_2)^2}$$

Substituting this value in equation (5)

$$O_2 = \frac{E_0 k_1 k_2 t}{k_1 + k_2} + \frac{E_0 k_1^2}{(k_1 + k_2)^2} (1 - e^{-\overline{k_1+k_2}t}) \quad (6)$$

This is the mathematical expression, according to these simple compensating reactions, for the amount of oxygen evolved at any time. It shows that the experimental curve can be considered in two parts, a straight line through the origin (first term equation (6)) and a curve which rises rapidly to a constant value for O_2 (second term, exponential, equation (6)). This is illustrated in Fig 11, which is based on Exp c in Fig 2. Curve A is the experimental curve, B the linear part and C the exponential part $A=B+C$

The amount of O_2 evolved in reaching the equilibrium ΔO_2 is given mathematically by the exponential term in equation (6) when $t=\infty$, and

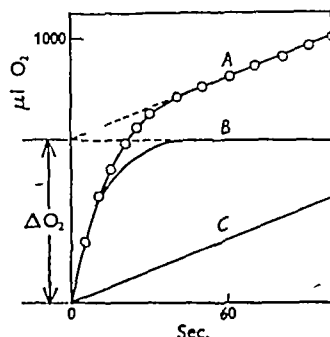


Fig 11 Analysis of experimental curve (Fig 2 c). Experimental curve, A. Linear portion, B. Exponential portion, C. $A=B+C$

experimentally by the extrapolation of curve A or C back to the ordinate, i.e.

$$\Delta O_2 = \frac{E_0 k_1^2}{(k_1 + k_2)^2}$$

Hence the relation between the oxygen evolved and the number of mol of catalyst present is

$$\frac{\Delta O_2}{E_0} = \left(\frac{k_1}{k_1 + k_2} \right)^2 \quad (7)$$

If the compensating reactions are slightly more complicated, and oxygen is evolved in both steps, it can be shown that

$$\begin{aligned} R_t &= \frac{E_0}{k_1 + k_2} [2k_1 k_2 + (k_1^2 - k_1 k_2) e^{-\overline{k_1+k_2}t}], \\ R_0 &= k_1 E_0, \\ R_e &= \frac{2E_0 k_1 k_2}{k_1 + k_2}, \\ \frac{\Delta O_2}{E_0} &= \frac{k_1 (k_1 + k_2)}{(k_1 + k_2)^2} \end{aligned}$$

Thus it is an essential feature of the mechanism based on compensating reactions that the ratio $\Delta O_2/E_0$ should be less than one

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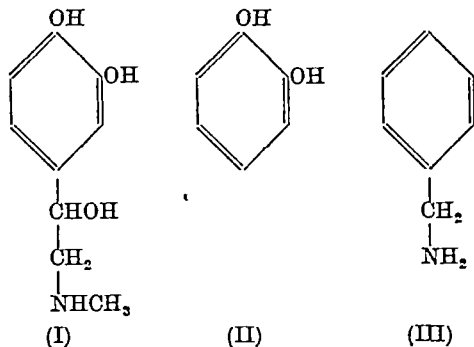
18 A STUDY OF THE RELATION BETWEEN CONJUGATION AND DEAMINATION OF *p* HYDROXYBENZYLAMINE AND RELATED COMPOUNDS IN THE RABBIT

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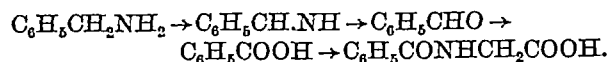
(Received 9 February 1948)

The present work was carried out as part of a programme of research on the inactivation of α adrenaline (I) in the body. There are two views of the mode of inactivation of adrenaline: briefly, the first is that the inactivation involves monoamine oxidase which oxidatively deaminates adrenaline, and the second is that the inactivation takes place through conjugation of the hydroxyl groups of adrenaline to produce an inactive ethereal sulphate (cf Richter, 1940, Richter & MacIntosh, 1941-2).

Our plan was to investigate the fate in the body of compounds containing the various structural elements of the adrenaline molecule. Our studies have included, for example, investigations on the metabolism of catechol (II) (Garton & Williams, 1948), *p*-hydroxybenzylamine (IV), *p*-hydroxybenzylmethylamine (V), protocatechuic acid (VI) and *D*-adrenaline (I) (Dodgson, Garton & Williams, 1947).



The fate of benzylamine (III) has been studied both *in vivo* and *in vitro*. It is oxidized *in vitro* by amine oxidase preparations from liver and intestinal tissue at about one tenth the rate at which tyramine is oxidized (Blaschko, Richter & Schlossman, 1937), and in the dog it is converted almost quantitatively into hippuric acid (Mosso, 1890), the mechanism of transformation (cf Green, 1941) being probably as follows



In *p*-hydroxybenzylamine (IV) two changes can take place *in vivo*, namely, conjugation of the OH group and oxidative deamination of the —CH₂NH₂

group, and it was our aim to find out how one process influenced the other. We shall show in this paper that conjugation of the OH group is related to the rate of deamination and oxidation of the —CH₂NH₂ group.

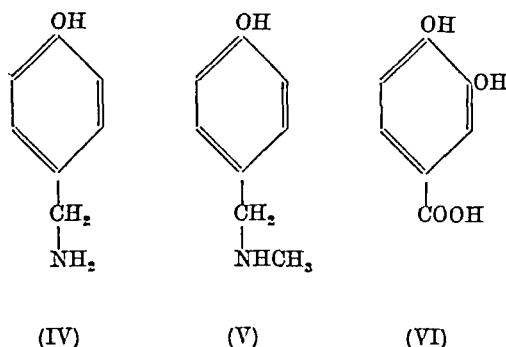
METHODS

Animals Chinchilla rabbits, 2-3 kg wt, on a diet of 50 g Lever's cubes and water *ad lib* were used throughout.

Analytical methods Etheral sulphate was determined by the Folin gravimetric method (cf Williams, 1938), glucuronic acid by the method of Hanson, Mills & Williams (1944), and free and combined *p* hydroxybenzoic acid by the method of Quirk (1932) which is based on the bromination method of Day & Taggard (1928).

MATERIALS

A *p* Hydroxybenzoic acid, m p 213° (all melting points are uncorrected), was prepared by hydrolysis of ethyl *p* hydroxybenzoate. B *p* Hydroxybenzaldehyde, m p 115°,



was a purified commercial sample. C *p* Hydroxybenzylamine was fed as the monohydrate, m p 98°, and prepared according to Tiffeneau (1911).

D *Preparation of p hydroxybenzylacetamide (N acetyl p hydroxybenzylamine)* This compound appears to be new. *p* Hydroxybenzylamine (5 g) was dissolved in a solution of 10 g K₂CO₃ in 25 ml water. 10 ml acetic anhydride were added slowly with shaking, the temperature being maintained below 5°. An oil separated which solidified on standing. The solid was broken up, washed with cold water, dried and recrystallized from benzene. *p* Acetoxybenzylacetamide, m p 62°, forms needles easily soluble in ethanol but insoluble in water (yield, 5 g) (Found C, 63.8, H, 6.45, N, 6.7. C₁₁H₁₃O₃N requires C, 63.75, H, 6.3, N, 6.8%). *p* Acetoxybenzylacetamide (5 g) was suspended in a solution of 5 g KOH in 50 ml water, and the mixture was refluxed gently until the solid dissolved. Heating was continued until

no oil globules separated on cooling. The solution was now cooled and acidified with HCl. On standing at 0° long white needles of *p*-hydroxybenzylacetamide separated. It was recrystallized from hot water (yield 3.5 g) and had *m* p 125°. It was slightly soluble in cold water but readily soluble in hot, and it gave a deep violet colour with FeCl₃. (Found C, 65.55, H, 6.8, N, 8.6. C₉H₁₁O₂N requires C, 65.4, H, 6.7, N, 8.5%)

E Preparation of *p* hydroxybenzylmethylamine The method of Tiffeneau (1911) for preparing this amine by heating anisyl chloride with methylamine in a sealed tube was found unsatisfactory owing to the formation of the tertiary di(*p* hydroxybenzyl)methylamine. We therefore devised a new synthesis.

A solution of 9 g anisylacetamide (*m* p 97°, Tiffeneau, 1911) in 100 ml dry benzene was refluxed for 3 hr with 4 g finely divided sodamide. The solution, now containing the sodio derivative of anisylacetamide, was cooled and treated with 8 ml methyl iodide. The mixture was gently refluxed for 10 min, then the NaI which separated was filtered off and the benzene removed by distillation *in vacuo*. The residual anisylmethylacetamide was a pale yellow oil (10 g). This oil was refluxed with 100 ml *n*-butanolic KOH for 2 hr to remove the acetyl group. The resulting solution was cooled, made strongly acid with conc HCl and then extracted with 3 × 25 ml portions of water. The aqueous extract, which contained anisylmethylamine hydrochloride, was made alkaline with solid KOH and extracted with ether. After drying the ethereal solution with solid KOH and filtering, the anisylmethylamine was precipitated as the hydrochloride by passing a stream of dry HCl through the solution. The yield of hydrochloride was 5 g, it was crystallized from ethanol-ether and had *m* p 165° (Tiffeneau (1911) gives *m* p 166°). Anisylmethylamine was converted into *p* hydroxybenzylmethylamine hydrochloride (*m* p 185°) according to Tiffeneau (1911).

RESULTS

A Experiments with *p* hydroxybenzoic acid

(1) *Ethereal sulphate and glucuronic acid outputs*
The figures in Table 1 indicate that about one quarter of the *p* hydroxybenzoic acid, fed at a dose level of 0.35 g/kg, is excreted conjugated through its hydroxyl group, and that the ratio glucuronic acid/etheral sulphate is about 2. Our figures are a little higher than those obtained in a more detailed study by Bray, Ryman & Thorpe (1947) for doses of 0.25 g/kg, but our results lead to essentially the same conclusion, viz. that the extent of conjugation

of *p* hydroxybenzoic acid through its hydroxyl group is in the region of 20–30% of the dose.

(2) *Isolation of *p* hydroxyhippuric acid* According to Bray *et al* (1947) about 20–30% of the dose of *p* hydroxybenzoic acid is conjugated with glycine. We did not repeat this estimation, but isolated the conjugated product.

A total of 6 g of *p* hydroxybenzoic acid was fed to 3 rabbits and a 24 hr urine (350 ml) collected. The basic lead acetate precipitate of the urine was prepared in the usual manner. This was suspended in water and the Pb removed with H₂S. The filtrate from PbS was continuously extracted with ether for 2 hr to extract *p* hydroxybenzoic and *p* hydroxyhippuric acids. On evaporating the ether a white crystalline residue remained. This residue was extracted with small portions of ether to remove *p* hydroxybenzoic acid and there remained 300 mg (3.6% of the dose) of *p* hydroxyhippuric acid, which after recrystallization from water had *m* p 238° (Bray *et al* (1947) and Fischer (1908) give *m* p 240°).

(3) *The glucuronide in *p* hydroxybenzoic acid urine*
Quick (1932) has reported the isolation, from the urine of dogs receiving *p* hydroxybenzoic acid orally, of *p* glucuronosidobenzoylglucuronide, a substance which in virtue of its ester glucuronide link reduces alkaline copper reagents. We have been unable to isolate such a glucuronide from rabbit urine and our evidence indicates that, if such a glucuronide is formed, then it must be present in very small amounts (cf. Bray *et al* 1947).

We found that *p* hydroxybenzoic acid urines from rabbits were non reducing, and conclude that the main glucuronide excreted is probably the non reducing *p* carboxyphenyl glucuronide. We were, however, unable to isolate this substance or a derivative of it in a crystalline state. It was obtained from the basic lead acetate precipitate of *p* hydroxybenzoic acid urine as a non reducing yellowish gum. Attempts to form a triacetyl methyl ester did not result in crystalline material. The glucuronide gum (0.5 g) was hydrolyzed by boiling with 15 ml of 3–4*N* HCl for 0.5 hr. Ether extraction of the hydrolysate yielded only *p* hydroxybenzoic acid, *m* p and mixed *m* p 213° (yield of pure material 0.1 g).

B Experiments with *p* hydroxybenzaldehyde

(1) *Ethereal sulphate and glucuronic acid outputs*
Table 2 shows that about 40% of the aldehyde is excreted conjugated through its hydroxyl group and

Table 1 *The ethereal sulphate and glucuronic acid conjugations of *p* hydroxybenzoic acid in the rabbit*

Rabbit no	Wt (kg)	Dose		Ethereal sulphate as SO ₃		Glucuronic acid		Dose excreted as		Total con jugation (%)
				Mean normal value (mg /day)	Increase after dosing (mg)	Mean normal value (mg /day)	Increase after dosing (mg)	Sul phate (%)	Glucu ronide (%)	
		(mg)	(mg /kg)							
68	2.9	1002	345	19.9	43.8	186.5	239.8	7.7	17.0	24.7
82	2.9	999	344	25.1	47.8	195.3	141.4	8.8	10.1	18.9
84	2.85	1009	253	16.6	66.2	148.6	377.1	12.1	26.6	38.7

the glucuronic acid/etheral sulphate ratio is about 3.5. The extent of total conjugation is greater than with *p*-hydroxybenzoic acid, the glucuronic acid conjugation of the aldehyde being twice that of the acid, although the sulphate conjugations are the same (cf Williams, 1938). Since *p*-hydroxybenzaldehyde is largely transformed *in vivo* into *p*-hydroxybenzoic acid (Quick, 1932, Dakin, 1910), it is highly probable, in view of the higher glucuronic

reddish orange microcrystalline solid, m.p. 166° (yield 125 mg). The compound gave positive tests for glucuronic acid, and after acid hydrolysis it gave a red colour for *p*-hydroxybenzaldehyde in the Sammons & Williams (1941b) colour reaction. These tests and elementary analysis indicated it to be *p*-glucuronosidobenzaldehyde 2,4-dinitrophenylhydrazone (Found C, 44.4, H, 4.2, N, 11.6. $C_{19}H_{18}N_4O_{11} \cdot 2H_2O$ requires C, 44.4, H, 4.3, N, 10.9%). A sample of this compound had been prepared by one of us in another investigation and it had m.p. 165° and mixed m.p.

Table 2 *The ethereal sulphate and glucuronic acid conjugations of p-hydroxybenzaldehyde in the rabbit*

Rabbit no	Wt (kg)	Dose		Ethereal sulphate as SO ₃		Glucuronic acid		Dose excreted as		Total con jugation (%)
				Mean normal value	Increase after dosing	Mean normal value	Increase after dosing	Sul phate	Glucu ronide	
		(mg)	(mg /kg)	(mg /day)	(mg)	(mg /day)	(mg)	(%)	(%)	
68	2.75	753	274	14.9	52.7	177.5	391.7	10.7	32.7	43.4
82	2.8	752	269	20.2	45.3	173.8	396.5	9.2	33.1	42.3
84	2.75	751	273	23.9	36.8	197.6	388.1	7.7	32.5	40.2

Table 3 *The ethereal sulphate and glucuronic acid conjugations of p-hydroxybenzylamine in the rabbit*

Rabbit no	Wt (kg)	Dose		Ethereal sulphate as SO ₃		Glucuronic acid		Dose excreted as		
				Mean normal output	Increase after dosing	Mean normal output	Increase after dosing	Ethereal sulphate	Glucu ronide	
		(mg)	(mg /kg)	(mg /day)	(mg)	(mg /day)	(mg)	(%)	(%)	
91	2.5	624	250	38.1	55.5	—	—	15.7	—	
92	2.5	644	258	23.6	80.8	—	—	22.1	—	
93	3.0	798	270	37.3	83.9	—	—	18.5	—	
99	2.7	663	250	—	—	225.9	406.6	—	44.6	
101	2.7	680	252	—	—	221.9	321.8	—	34.4	
104	2.5	622	250	—	—	184.0	323.5	—	38.4	
							Average	18.8	39.4	
							Average total conjugation			58.2

acid excretion provoked by the aldehyde, that some *p*-hydroxybenzaldehyde is conjugated with glucuronic acid before its aldehyde group becomes oxidized. In an earlier paper on vanillin (Sammons & Williams, 1941a) it was shown that the aldehyde glucuronide could be detected in urine in small amounts during the early hours after dosing.

(2) *Isolation of p-glucuronosidobenzaldehyde 2,4-dinitrophenylhydrazone* *p*-Hydroxybenzaldehyde (8 g) was fed to 4 rabbits, and the urine was collected for the following 5 hr. The urine gave a red colour with naphthoresorcinol and HCl in the cold, thus indicating that either *p*-hydroxybenzaldehyde or an *O*-conjugated aldehyde was being excreted (colour reaction of Sammons & Williams, 1941b). The urine (300 ml) was filtered first through cotton wool and then through filter paper, and to it was added 15 ml of conc. HCl followed by a solution of 1 g 2,4-dinitrophenylhydrazine in 15 ml conc. HCl and 100 ml ethanol. The mixture was kept at 0° overnight, during which time an orange-red precipitate separated. More ethanol was added and the precipitate was filtered and washed with dilute HCl, water and finally ethanol (yield 0.3 g). The solid was extracted with hot ethanol, and on cooling the extracts there was deposited a

with the above sample 166° (Found C, 44.5, H, 4.4, N, 11.3%).

The yield of the hydrazone corresponded to about 2–3% (6% in one case) of the aldehyde fed. None of the conjugated aldehyde could be detected in the urines collected later than 5 hr after feeding.

C. Experiments with *p*-hydroxybenzylamine

(1) *The ethereal sulphate and glucuronic acid conjugations* The results are given in Table 3 which shows that about 58% of the *p*-hydroxybenzylamine is excreted as *O*-conjugates and that the ratio glucuronic acid/etheral sulphate is roughly 2. The glucuronic acid conjugation (39%) is slightly higher than that for *p*-hydroxybenzaldehyde (33%), but the ethereal sulphate conjugation (19%) is double that of both *p*-hydroxybenzoic acid and aldehyde (9%). These results could be interpreted as meaning that the amine is partly sulphated before deamination, but conjugated with glucuronic acid after conversion to the aldehyde but before conversion to the acid.

(2) *Isolation of p hydroxybenzoic and p hydroxyhippuric acids* Three rabbits were each given 2 g *p* hydroxybenzylamine with water by stomach tube. The 24 hr urine (390 ml) was acidified with 40 ml of conc HCl, filtered through glass wool and then extracted with ether continuously for 3 hr. The extract was evaporated to a syrup which crystallized on adding 10–15 ml of water. The crystals were filtered off, and after two recrystallizations from water yielded white plates of *p* hydroxybenzoic acid, *m* p 212° and mixed *m* p 213°. The yield after recrystallization was 1 g or 17% of the dose of amine. This material represents the free acid excreted.

The combined *p* hydroxybenzoic acid was obtained by continuous ether extraction for 3 hr of the residual urine, which had been brought to pH 1 and boiled under reflux for 1 hr. The yield of combined acid (*m* p and mixed *m* p 213°) was 1.1 g or about 18% of the dose of amine.

For the isolation of *p* hydroxyhippuric acid, a 24 hr urine, after the feeding of 6 g of *p* hydroxybenzylamine, was acidified and extracted with ether as described above. The ether extract was evaporated and the residue extracted with small portions of ether. Most of the residue dissolved, leaving a small residuum of *p* hydroxyhippuric acid (100 mg, or 1% of the dose of amine). On recrystallization from hot water the acid formed plates, *m* p and mixed *m* p 238°. These isolation experiments account for nearly 40% of the amine fed.

an equal volume of conc HCl (Sammons & Williams, 1941b). The colour increases in intensity in the course of 1 hr and thereafter it fades to a dirty brown colour. This colour reaction suggests that a conjugate of *p* hydroxybenzaldehyde is being excreted. An attempt was made, after feeding 8 g of the amine, to isolate the aldehyde as a 2,4-dinitrophenylhydrazone according to the method already described under the section B(2) on *p* hydroxybenzaldehyde. A small amount (15 mg) of a hydrazone was obtained, but we were unable to identify it as *p* glucuronosidobenzaldehyde 2,4-dinitrophenylhydrazone. The material melted indefinitely, but gave a deep purple colour in dilute NaOH similar to that given by the authentic aldehyde derivative. It also gave the Tollens reaction for glucuronic acid.

In another experiment a 24 hr urine, after feeding 6 g of the amine, was collected and the basic lead acetate precipitate was prepared in the usual manner. The Pb was removed with H₂S and the excess H₂S in the filtrate removed by aeration. The filtrate gave a pale red colour in the Sammons & Williams test, but after gentle hydrolysis with HCl a strong red colour was obtained indicating the presence of *p* hydroxybenzaldehyde. Attempts to prepare an identifiable 2,4-dinitrophenylhydrazone on half of the filtrate failed. The other half was hydrolyzed by boiling for 1 hr with an equal volume of conc HCl. The dark solution was cooled, decolorized with charcoal and filtered. This solution gave an intense red colour in the Sammons & Williams

Table 4 *Free and conjugated acid (calculated as p hydroxybenzoic acid) excreted by rabbits receiving p hydroxybenzylamine*

		Acid excreted (calculated as <i>p</i> hydroxybenzoic acid)											Total acid – free acid = conjugated acid (%)		
		Blank value on urine before hydrolysis (mg)	Free acid					Blank value on urine after hydrolysis (mg)	Total acid						
			Day 1		Day 2		Total		Day 1		Day 2			Total	
Rabbit no	Dose of amine (mg)		(mg)	(% of dose)	(mg)	(% of dose)			(% of dose)	(mg)	(% of dose)	(mg)			(% of dose)
Wt (kg)															
91	2.5	624	136.5	163	26.7	115	18.8	45.5	118.6	456.3	74.7	114.8	18.8	93.5	48
92	2.5	644	133.1	260.3	41.3	53.3	8.5	49.8	122.6	494.7	78.5	110.8	17.6	96.1	46.3
93	3.0	798	174.1	139.6	17.9	101	12.6	30.5	143.6	498	63.7	119.5	15.3	79.0	48.5

(3) *Quantitative determination of free and combined p hydroxybenzoic acid* The results are given in Table 4 which show that about 90% of the dose of *p* hydroxybenzylamine is excreted as *p* hydroxybenzoic acid. The figures for free *p*-hydroxybenzoic acid include also any *p* hydroxyhippuric acid, and give, therefore, a measure of the amount of *p* hydroxybenzylamine excreted as acidic compounds carrying a free phenolic hydroxyl group, i.e. about 40%. The amount of *p* hydroxybenzylamine excreted as *O* conjugates is by this method about 47.5%, which is somewhat lower than that found by summation of the glucuronic acid and ethereal sulphate conjugations, 58.1%, given in Table 3. The values obtained by these two methods are, however, of the same order.

(4) *Detection of p hydroxybenzaldehyde derivatives* The urine collected up to 6 hr from rabbits after their receipt of doses of *p* hydroxybenzylamine gives a faint red colour when treated with a little naphthoresorcinol in ethanol and

reaction, an intense Tollens reaction and reduced Benedict's reagent. It was extracted with ether to remove any *p* hydroxybenzaldehyde present. The ether extract was taken to dryness and the residue taken up in 10 ml ethanol. This ethanolic solution gave a very intense red colour in the Sammons & Williams test, and was treated with 2,4-dinitrophenylhydrazine in ethanol containing H₂SO₄. A small red precipitate, *m* p 270° (after recrystallization from ethanol/benzene), separated. We were unable, however, to identify it definitely as *p* hydroxybenzaldehyde 2,4-dinitrophenylhydrazone (*m* p 280°) for the quantity obtained was too small for micro analysis.

We conclude from these experiments that it is very probable that a small amount of conjugated *p* hydroxybenzaldehyde is excreted after *p*-hydroxybenzylamine has been fed to rabbits, but we have been unable finally to prove this by actual isolation of the aldehyde.

(5) *Detection of nuclear oxidation of p hydroxybenzylamine in vivo* Bray *et al* (1947) have shown that *p* hydroxybenzoic acid is oxidized to a small

extent to protocatechuic acid. They were unable to isolate this acid after feeding *p*-hydroxybenzoic acid, but did so when *p*-hydroxybenzamide was fed. It is therefore possible that, since *p*-hydroxybenzylamine is mainly converted to *p*-hydroxybenzoic acid, the latter may also be slightly oxidized to protocatechuic acid.

p-Hydroxybenzylamine (12 g) was fed to 6 rabbits and a 24 hr urine (600 ml.) collected. The urine was acidified with HCl and extracted continuously with ether for 3 hr. Removal of the ether left a crystalline mass, which on recrystallization from water yielded *p*-hydroxybenzoic acid, m.p. 214°. The mother liquors gave no green colour, under the appropriate conditions, with FeCl₃, indicating the absence of any free protocatechuic acid. The urine was now made strongly acid with conc. HCl and boiled for 1 hr. It was then cooled and extracted continuously for 3 hr with ether. Removal of the ether and recrystallization of the residue from water yielded *p*-hydroxybenzoic acid. The mother liquor now gave an intense green colour with FeCl₃, which turned blue, violet and finally red on adding NaHCO₃. We were unable to isolate crystalline protocatechuic acid from this mother liquor, but there is little doubt that it was present.

We therefore conclude that *p*-hydroxybenzylamine is converted to a very small extent to a catechol derivative which is probably conjugated protocatechuic acid.

(6) *Observations on the glucuronide of p-hydroxybenzylamine urine*. The glucuronide of *p*-hydroxybenzylamine urine was obtained via the basic lead acetate precipitate as a gum. Attempts to form crystalline derivatives such as salts or the triacetyl methyl ester were not successful (see section A(3)). The gum was a non-reducing acidic substance, and on acid hydrolysis yielded *p*-hydroxybenzoic acid, m.p. 213°.

We conclude that the glucuronide excreted after feeding *p*-hydroxybenzylamine is largely *p*-carboxyphenylglucuronide.

D Experiments with *p*-hydroxybenzylacetamide

(1) *Ethereal sulphate and glucuronic acid excretion*. Table 5 shows that about 70–80% of *p*-hydroxybenzylacetamide is excreted as *O* conjugates, the glucuronic acid/ethereal sulphate ratio being about 5

(2) *The isolation of unchanged p-hydroxybenzylacetamide*. A rabbit was fed 2 g of *p*-hydroxybenzylacetamide and a 24 hr urine collected. The urine was made acid to Congo red and continuously extracted with ether for 3 hr. The ether was removed and the residue recrystallized (charcoal) from hot water. The crystals (25 mg) obtained were identified as *p*-hydroxybenzylacetamide, m.p. and mixed m.p. 125°. No trace of *p*-hydroxybenzoic acid was found, and there was no evidence of deacetylation.

(3) *The glucuronide of p-hydroxybenzylacetamide*. Each of 3 rabbits was fed 1 g of the amide and a 6 hr urine (110 ml) collected. Very little glucuronide was excreted after the first 6 hr. The basic lead acetate precipitate was prepared in the usual manner and the Pb removed by H₂S. The filtrate from PbS was treated with charcoal, filtered and concentrated to 25 ml. Addition of ethanol to this solution precipitated the glucuronide as an amorphous solid which became gummy on standing. It was therefore redissolved by addition of more water, and the whole was evaporated under reduced pressure to a hard dry amorphous powder (1.5 g). It could not be induced to crystallize.

The powder was shaken for 10 min with an ethereal solution of diazomethane (from 1.5 g of nitrosomethylurea). It formed a gum on the sides of the flask and the mixture was kept at room temperature overnight. During this time the colour of the diazomethane had disappeared, and a further quantity of ethereal diazomethane together with 5 ml of ethanol were added. The mixture was kept for 48 hr, then decanted from any solid material and taken to dryness at 40°. The residue did not crystallize. It was therefore dissolved in 5 ml pyridine and 5 ml acetic anhydride and kept overnight at room temperature. The mixture was then poured into 50 ml of water and the solution neutralized with solid Na₂CO₃. On standing for 5–6 days, the solution deposited rosettes of fine needles. These were collected and recrystallized from acetone-water (yield 100 mg). The product, *p*-acetamidomethylphenyl triacetylglucuronide methyl ester, formed rosettes of needles, m.p. 174°, and showed $[\alpha]_D^{20} -29.05^\circ$ ($c=1.8$ in acetone) (Found C, 54.95, H, 5.9, N, 3.2. C₂₂H₂₇O₁₁N requires C, 54.9, H, 5.65, N, 2.9%).

E Experiments with *p*-hydroxybenzylmethylamine

(1) *Ethereal sulphate and glucuronic acid conjugations*. Table 6 shows that about 60% of the amine fed is excreted as *O* conjugates, the ratio glucuronide/ethereal sulphate being about 8. It is to be noted that the sulphate conjugation is relatively low (7%) and is only a third of that of

Table 5 *The ethereal sulphate and glucuronic acid conjugation of p-hydroxybenzylacetamide in the rabbit*

Rabbit no	Wt (kg)	Dose		Ethereal sulphate as SO ₃		Glucuronic acid		Dose excreted as		Total con jugation (%)
				Mean normal value (mg/day)	Increase after dosing (mg)	Mean normal value (mg/day)	Increase after dosing (mg)	Sul phate (%)	Glucu ronide (%)	
		(mg)	(mg/kg)							
99	2.7	610	230	32.2	46.5	202	481	15.7	67.0	82.7
101	2.7	630	233	26.8	21.7	184	657	7.1	88.7	95.8
104	2.5	504	201	35.3	31.7	220	361	13.1	61.0	74.1
110	3.0	795	253	16.3	57.8	153	475	15.7	53.2	68.9
111	3.2	747	234	15.3	51.8	96	465	14.3	52.0	66.3
112	2.9	745	261	15.5	42.2	134	272	11.7	39.1	50.8

p hydroxybenzylamine (19%) This result is similar to that found for *D* adrenaline (Dodgson *et al* 1947) On the other hand, the glucuronic acid conjugation of this amine (54%) is higher than that of *p* hydroxybenzylamine (39%), and is approaching that of *p* hydroxybenzylacetamide (60%)

The main metabolites are free *p* hydroxybenzoic acid, its ethereal sulphate and ether glucuronide Small but isolable amounts of *p* hydroxyhippuric acid and traces of conjugated *p* hydroxybenzaldehyde and conjugated protocatechuic acid are also excreted

Table 6 The ethereal sulphate and glucuronic acid conjugations of *p* hydroxybenzylmethylamine hydrochloride in the rabbit

Rabbit no	Wt (kg)	Dose (mg) (mg/kg)		Ethereal sulphate as SO ₃		Glucuronic acid		Dose excreted as	
				Mean normal value (mg/day)	Increase after dosing (mg)	Mean normal value (mg/day)	Increase after dosing (mg)	Sulphate (%)	Glucuronide (%)
109	2.5	601	245	28.3	19.7	130	360	7.1	52.4
115	2.6	603	232	27.6	22.2	134	342	8.0	49.6
116	2.6	604	232	19.8	18.9	127	323	6.8	46.7
110	3.0	1474	499	17.5	35.6	137	979	5.2	59.4
111	2.8	1474	526	13.3	54.8	127	1100	7.9	64.1
112	3.2	1500	476	14.2	31.9	62.4	850	4.7	50.5

(2) Isolation of *p* hydroxybenzoic acid after feeding *p* hydroxybenzylmethylamine Each of 3 rabbits received 1.5 g of the amine hydrochloride and a 24 hr urine (1100 ml) was collected From 400 ml of the acidified urine 0.1 g of *p* hydroxybenzoic acid, m.p. 214°, was isolated by ether extraction The extracted urine was strongly acidified with conc. HCl and refluxed for 1 hr Ether extraction of the hydrolyzed urine yielded 0.1 g *p* hydroxybenzoic acid, m.p. 214°

Thus 45% of the amine fed was accounted for by isolation as *p* hydroxybenzoic acid, half of the acid occurring in the urine as an *O* conjugate and the other half free This experiment indicates that *p* hydroxybenzylmethylamine is excreted largely as free and conjugated *p* hydroxybenzoic acid

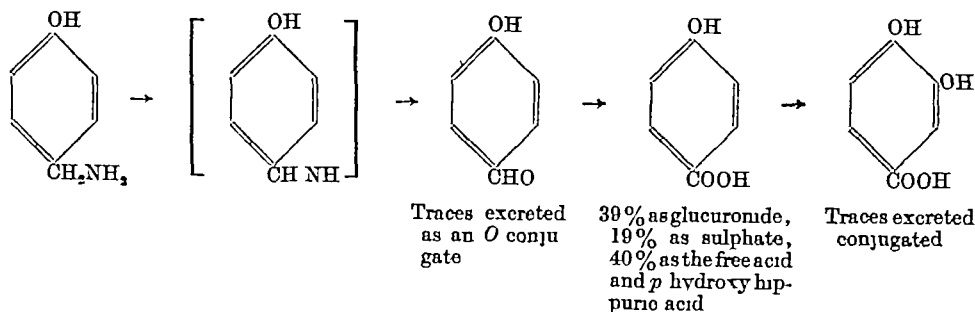
DISCUSSION

The ethereal sulphate and glucuronic acid conjugations of the five compounds studied are summarized in Table 7 (p. 302)

The fate of *p* hydroxybenzylamine in the rabbit can be expressed as follows

The metabolism of *p* hydroxybenzylamine involves two separate mechanisms (a) deamination followed by oxidation of the —CH₂NH₂ group, and (b) conjugation of the phenolic hydroxyl group It is interesting, therefore, to consider how the one mechanism affects the other The deamination process can be blocked by acetylating the amino group as in *p* hydroxybenzylacetamide, and from the figures (see Table 7) for the conjugation of this amide it is clear that when deamination does not take place there is a high *O* conjugation (73%) particularly with glucuronic acid (60%)

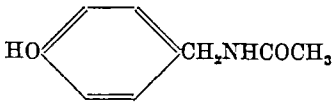
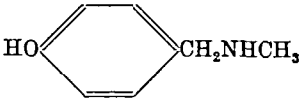
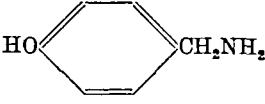
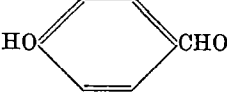
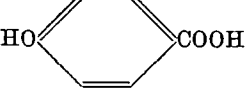
p Hydroxybenzylmethylamine is intermediate between *p*-hydroxybenzylamine and *p* hydroxybenzylacetamide in the extent of its conjugation, and this suggests that it is more slowly deaminated than the primary amine Its rate of deamination is such as to allow a considerable amount of *O* conjugation to take place This result indicates that secondary amines of the type *R* CH₂NHCH₃ are more slowly deaminated *in vivo* than the corresponding primary amines, *R* CH₂NH₂ This conclusion receives support from the earlier findings of



Ewins & Laidlaw (1910) and of Alles & Heegaard (1943) Ewins & Laidlaw showed that tyramine (*p*-hydroxyphenylethylamine) was readily converted to *p* hydroxyphenylacetic acid in the perfused dog liver, whereas *p* hydroxyphenylethyl methylamine formed the acid at a much slower rate. Hordenine (*p*-hydroxyphenylethyl dimethylamine) only formed traces of *p* hydroxyphenylacetic acid after prolonged perfusion. Alles & Heegaard studied *in vitro* the rate of deamination by amine oxidase of derivatives of some sympathomimetic amines, and found the *N*-

aldehyde in the wall of the gut, before being conjugated with glucuronic acid in the liver. Furthermore, this conjugation is virtually complete before the aldehyde is oxidized to *p* hydroxybenzoic acid, for the glucuronic acid conjugation of this acid (18%) is much lower than that of the aldehyde. The sulphate conjugation of the amine (19%), on the other hand, is much higher than that of the aldehyde (9%), and this may indicate that some sulphate conjugation of the amine occurs in the wall of the gut before it is deaminated to the aldehyde.

Table 7 The O conjugation of *p* hydroxybenzylamine and related compounds in the rabbit

Compound	% of compound excreted as		Total O conjugation (% of dose)	Extent of conversion to <i>p</i> hydroxybenzoic acid
	Ethereal sulphate	Glucuronide		
	13	60	73	Nil
	7	54	61	Probably complete
	19	39	58	Complete
	9	33	42	Complete
	9	18	27	—

monomethyl derivatives to be more slowly deaminated than the parent amines. Our results suggest that some idea of the rate of the *in vivo* deamination of phenolic amines may be obtained from a determination of their glucuronic acid conjugations, the possible application of this suggestion to adrenaline is obvious.

Table 7 shows that the glucuronic acid conjugations of the compounds studied form a regular series, but this is not true for the sulphate conjugation. However, sulphate conjugation is complicated by the fact that it occurs in the intestine as well as in the liver (Marenzi, 1931, Arnoldt & De Meio, 1941), whereas the glucuronic acid conjugation occurs only in the liver. It is not possible, therefore, to comment at this stage on the sulphate conjugation of these compounds.

The similarity between the extent of the glucuronic acid conjugation of *p* hydroxybenzylamine (39%) and of *p* hydroxybenzaldehyde (33%) suggests that after being fed the amine is largely converted to the

SUMMARY

1 The metabolic fates in the rabbit of *p*-hydroxybenzylamine, *p* hydroxybenzylmethylamine, *p*-hydroxybenzylacetamide, *p* hydroxybenzaldehyde and *p* hydroxybenzoic acid have been compared.

2 The main metabolic products of *p* hydroxybenzylamine are free *p* hydroxybenzoic acid (c 40%) and the glucuronide (39%) and ethereal sulphate (19%) of *p* hydroxybenzoic acid. Small amounts of conjugated *p* hydroxybenzaldehyde, *p*-hydroxyhippuric acid and conjugated protocatechuic acid are also excreted.

3 *p* Hydroxybenzylmethylamine is also converted into *p* hydroxybenzoic acid derivatives, but in this case the glucuronide formed is 54% of the dose whereas the sulphate is 7%.

4 *p* Hydroxybenzylacetamide is not deacetylated *in vivo* and does not produce derivatives of *p* hydroxybenzoic acid in the urine. It is mainly transformed (60%) into *p* acetamidomethylphenylglu-

curonide which has been isolated and characterized as its triacetyl methyl ester *p* Hydroxybenzylacetamide and its *O* acetyl derivative have been prepared and described for the first time

5 *p* Hydroxybenzaldehyde is largely converted into the glucuronide (33%) and sulphate (9%) of *p* hydroxybenzoic acid. A small amount (2-3%) is, however, excreted as *p* glucuronosidobenzaldehyde which has been isolated as a 2,4 dinitrophenylhydrazine

6 *p* Hydroxybenzoic acid is the least conjugated

of all the compounds studied here and forms only 18% glucuronide and 9% ethereal sulphate

7 The results indicate that with phenolic amines of the type studied here the extent of glucuronic acid conjugation is inversely proportional to the rate of deamination, the conjugation being highest where no deamination takes place. The significance of this conclusion is discussed

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Further Observations on the Proteolytic Enzymes in Rat Skin

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The object of this communication is to extend knowledge of the skin proteinase or 'dermoproteinase' previously described by Beloff & Peters (1945) and of its distinction from other proteolytic enzymes present in rat skin (see especially Fruton, 1946)

It was shown that the dermoproteinase will digest casein, serum globulin, serum albumin and myogen, and that it does not fall within the known groups of digestive proteinases by virtue of its failure to digest *N*-benzoyl-L-arginine amide and carbobenzyloxy-L-tyrosylglycine amide, the typical synthetic substrates for trypsin and chymotrypsin. Beloff & Peters (1945) also showed that this proteinase activity could be separated from other peptidases present by differential extraction of the skin.

The experimental results to be presented here will be considered under the separate headings (1) of the effect of methods of extraction upon the enzymes present, (2) of the character of the individual enzymes.

METHODS

Preparation of skin

(a) Acetone-dried rat skin was made as described previously (Beloff & Peters, 1945), 0.3 g. of dried skin being equivalent to 1.0 g. of fresh skin. Extracts were prepared so that 5 ml. of extract were equivalent to 0.3 g. of dried skin, either 5% (w/v) aqueous KCl or 5-6% (w/v) aqueous KNO₃ (see below) were used for the extraction of the proteinase.

(b) Fresh skin extracts were made by the technique of Fruton (1946). About 30 g. of skin (from two rats), carefully cleaned, were cut into small pieces and stirred with 100 ml. 2% (w/v) NaCl solution in the Waring Blendor for 8-10 min. A further 100 ml. of 2% NaCl were then added, and the whole stirred slowly for 3 hr. at room temperature. The pulpy mass of skin was strained off through muslin, and the extract filtered through a Whatman no. 41 filter paper. Certain comparisons between the extracts obtained by the two methods are given below.

Estimation of activity

The principle was followed throughout of mixing equal volumes of extract and substrate

(a) The peptidase activity was estimated by the titration technique of Linderström Lang (1927) in 90% (v/v) acetone. That is to say, acetone was added to the aqueous solution to be titrated until the liquid in the titration flask contained 90% acetone, and the titration was then carried out with ethanolic HCl (a standard solution of HCl in acetone being impracticable owing to the volatility of acetone). The synthetic peptide substrates were made up in the required concentration in distilled water, or in veronal buffer of the required pH, according to the extract being used. A 2 hr digestion period was normally allowed, duplicate 1 ml samples being taken at the beginning and end of this time. The samples were run into a calculated quantity of 0.1N ethanolic HCl, and the titration was completed with 0.01N ethanolic HCl. A difference of 0.03 ml or less (rarely 0.04 ml) between titration of duplicates was observed.

The synthetic peptides used were supplied commercially by Hoffmann-La Roche and Co. Except in two experiments (Table 5) the racemic, DL peptides were used, a control experiment indicated that the presence of the D peptide exerted no inhibitory effect (see also Beloff & Peters, 1945, addendum).

(b) The proteinase activity was estimated by the Van Slyke method. An attempt was made to use the Linderström-Lang titration for this, but it was found unsuitable for the following reasons. Casein, the substrate used, cannot be satisfactorily titrated, since it is precipitated on addition of acetone, the precipitation becoming heavier as the titration proceeds, and being apparently complete at the end point, thus rendering the colour change very difficult to observe. This was assumed to be due to the fact that the pH at the end point (c. 4) is approximately the same as at the isoelectric point of casein (4.7). β -Lactoglobulin and serum albumin were satisfactorily titrated, but it was discovered that concentration of salts also affects the accuracy of the titration. Phosphate in particular is precipitated very readily on addition of acetone, giving a rather cloudy effect, and causing a continuous shift of the end point. With 0.1M phosphate buffer, titration was not complete for many hours. This effect varies markedly with the salts present, and a solution containing 1% chloride was readily titratable, test-tube experiments indicating that up to 5% chloride would probably be satisfactory. Solutions containing 6% KNO_3 were also satisfactorily titrated. The combination of the effects of protein precipitation and of salts made the titration too uncertain to be useful in this instance.

The presence of 2.5% KCl in the digestion mixture was found to have an unfavourable effect on the Van Slyke method, a grey scum forming in the chamber, and the mercury losing its characteristic physical properties, becoming dull and sticky. This phenomenon was known previously but became in these experiments too troublesome to manage. It was found to be due to a reaction which took place between chloride, nitrite, and mercury in the presence of acid (simplest conditions, HCl, nitrite and mercury). Bromide had a still more marked effect than chloride. The occurrence of the reaction was confirmed using the same amounts of the reagents (A.R.) as were present during our

estimation, with a specimen of chemically purified, vacuum-distilled mercury. This led to an attempt to use other salts for the extraction of the skin: a comparatively inactive extract was obtained with sodium acetate and K_2SO_4 , whereas 6% (w/v) KNO_3 extracted about two thirds as much activity as 5% KCl. Two experiments are given in Table 1 comparing the extracting activity for dermoproteinase of 5% KCl and 6% KNO_3 . In the later experiments 6% KNO_3 was used.

Table 1 Comparison of 5% KCl and 6% KNO_3 as extracting agents for dermoproteinase from skin

(Dried skin (0.3 g) extracted for 30 min at room temperature with 5 ml extraction medium and extract mixed with an equal volume of substrate solution (0.5% casein). Digested at 38° for 2 hr. Proteinase activity estimated by Van Slyke method.)

Exp no.	Agent	Amino N (mg/100 ml)	
		Initial	Increase
1	KCl	0.813	0.379
	KNO_3	0.813	0.314
2	KCl	0.861	0.355
	KNO_3	0.888	0.281

Light white soluble casein (British Drug Houses Ltd.) was used, 1% solutions were made up directly in the Waring Blendor with the addition of a little NaOH, the pH being adjusted to the required point with 0.1N HCl.

The usual period of digestion of casein solutions and enzyme was 1 hr, unchanged protein being precipitated at the end of that time with 2 ml. 25% (w/v) trichloroacetic acid. The precipitates were washed with 2.5% trichloroacetic acid and the filtrates were made up to 25 ml., 5 ml samples being estimated.

Control tubes, to which trichloroacetic acid was added immediately after mixing the casein solution and extract, were treated in exactly the same way.

RESULTS

(1) Effects of methods of extraction upon the enzymes present

(a) *Dried skin extracts*. Extracts of acetone dried rat skin were previously shown to contain, in addition to the skin proteinase, some peptidase activity for leucylglycylglycine. The peptidase activities were extracted by a lower salt concentration. This was confirmed. Even 0.05M-phosphate extracted as much or slightly more peptidase than was present in a subsequent extraction of the same skin with addition of 6% nitrate, in contrast to this the higher salt concentration brought out much more proteinase (see Table 2). These extracts also contained some aminooxopeptidase activity, splitting leucylglycine with activation by manganese, though both peptidase activities were weak compared with those of Fruton-type extracts of fresh skin (see Table 3).

Table 2 *Influence of salt concentration upon the extraction of proteases from dried skin*

(Skin extracted as described in Table 1 and extract mixed with equal volume of substrate solution Digested at 38° for 2 hr Peptidase activity estimated by Linderström Lang titration, proteinase activity by Van Slyke method.)

Peptidase activity (substrate, DL-leucylglycylglycine)	
Extraction medium	Substrate split (mg /ml.)
1st extraction, 0.05 M Ringer phosphate	0.17*
2nd extraction, 0.05 M phosphate + 6% KNO ₃	0.11

Proteinase activity (substrate, 0.5% casein)		
Extraction medium	Amino N (mg /10 ml.)	
	Initial	Increase
1st extraction, 0.05 M Ringer phosphate	1.671	0.102
2nd extraction, 0.05 M phosphate + 6% KNO ₃	1.851	0.207

* The concentrations of substrate were not the same in these two experiments, and this figure has been corrected from the curve given (see Fig. 1) to be directly comparable with the other

Table 3 *Comparison of peptidase activity in different extracts of rat skin*

(Extracts of dried skin made as described in Table 1 Fruton type extracts made as described under Methods, 1 (b) Digestion for 2 hr at 38° Estimations by Linderström Lang titration)

Extraction medium	Substrate	Substrate concentration (mg /ml)	pH of digest	Substrate split (mg /ml)	
				0.001 M MnSO ₄	No activator
Acetone dried skin extracts					
2 % NaCl	DL-Leucylglycine	9.4	7.9	0.61	0.26
	DL-Leucylglycine	9.4	7.9	0.33	0.22
	DL-Leucylglycine	9.4	7.9	0.88	0.29
	DL-Leucylglycylglycine	24.5	7.9	0.27	0.19
Fruton type extracts					
2 % NaCl	DL-Leucylglycine	9.4	7.9	2.73	0.56
	DL-Leucylglycylglycine	24.5	7.9	3.13	1.52

Table 4 *Test for presence of additional activator in skin*

(Three tubes containing equal amounts of DL-leucyl glycine digested for 2 hr each, (a) with a mixture of an acetone-dried skin extract and a fresh (Fruton type) extract, (b) with the dried skin extract alone, and (c) with the fresh skin extract alone The quantity of each extract separately was the same as that present in the mixture of the two, volumes being made up with distilled water)

Extract	Substrate concentration (mg /ml)	Leucylglycine split (mg /ml.)
Mixed	9.4	0.87
Fresh skin alone	9.4	0.56
Dried skin alone	9.4	0.35

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It was thought possible that an additional activator, as well as the manganese, was present in the fresh skin extracts, but was partly destroyed by the acetone treatment, this was tested in two experiments In the first, a normal extract of dried skin was made with 2% NaCl and portions of this extract were used for a digestion of DL leucylglycine To other portions were added small quantities of another very concentrated extract, which had previously been boiled to destroy the enzyme activity, and these were then used for similar digestions The results were, within limits of experimental error, purely additive, and it was concluded that no heat stable activator was present

In the second experiment, samples of DL leucylglycine were digested (1) with a mixture of the two extracts (dried skin extract and Fruton type extract), (2) with the same volume of dried skin extract as used in the mixture, and (3) by the same volume of fresh skin extract as used in the mixture Results are given in Table 4

The effect, within the limits of experimental error, was again purely additive, and it was concluded that no additional activator was present, the difference between the two extracts being presumably due to the effect of the acetone treatment on the peptidase activity

It is of interest in this connexion to note that Maschmann (1942), working on glycerol extracts of kidney, liver and other tissues, found that desiccation with acetone caused substantial losses in peptidase activity The enzyme activity most affected was the cleavage of alanyl-glycylglycine and glycylglycylglycine, while that of leucylglycylglycine and leucylglycine was only slightly altered

(b) *Fresh skin extracts* Some of Fruton's results were confirmed The crude extracts contained the aminoexopeptidase and dermo-peptidase (see Table 3) The former was found to be present in larger proportion, the latter in lower proportion than found by Fruton in rabbit skin The two results are compared in Table 5

Table 5 *Comparison of amounts of peptidase present in rat and rabbit skin*

(The activity of Fruton type extracts of rat skin compared with Fruton's results on rabbit skin)

Skin	Substrate	Concentration of L-substrate (mg/ml)	pH	Time (hr)	Percentage hydrolysis	
					No activator	0.001 M MnSO ₄
Rat	DL-Leucylglycylglycine	12.25	7.9	2	12.4	25.6
	DL-Leucylglycylglycine	12.25	7.9	2	17.6	30.1
Rabbit (Fruton)	L-Leucylglycylglycine	12.25	7.9	2	24.0	38.0
Rat	L-Leucylglycine	9.4	7.9	2	19.5	78.5
	L-Leucylglycine	9.4	7.9	2	8.4	54.1
Rabbit (Fruton)	L-Leucylglycine	9.4	7.9	5	3.0	41.0

The proportionately greater effect of manganese in the case of the leucylglycylglycine splitting activity of rat skin, as opposed to rabbit skin, is presumably due to the greater proportion of the leucine aminopeptidase present.

It was confirmed that dialysis had the effect, reported by Fruton, of removing the leucine aminopeptidase activity, leaving the leucylglycylglycine splitting activity which is not activated by manganese, though, in the present work, a longer period of dialysis was necessary. The dermopeptidase activity against leucylglycylglycine was also reduced, apparently to an extent greater than proportional to the dilution occurring during dialysis. A volume of 26 ml of the crude extract was dialyzed for 3 days (Fruton's time) at about 1° against 1500 ml of distilled water. 29 ml of extract were

collected from this dialysis, and it was then found that the leucylglycylglycine splitting activity had been reduced by half, the leucylglycine splitting activity by only one third. The remaining extract was dialyzed against 4 l distilled water for a further 12 hr, during which time no further change in volume occurred. It was then found that the leucylglycylglycine splitting activity had remained constant, while the leucylglycine splitting activity had fallen markedly, though it was still not entirely removed. The results are given in Table 6.

The fresh skin extract was found to contain the dermoprotease, though the amount was low compared with that in the dried skin extracts. Hence this enzyme is again proved to be distinct from the other two. Results are given in Table 7.

Table 6 *Effect of dialysis upon the peptidases of rat skin*

(Fruton type extract dialyzed twice, once for 3 days, then again for 12 hr (see text). Activities against leucylglycine and leucylglycylglycine are given after each dialysis for comparison with original values.)

Dialyzed	Substrate	Substrate concentration (mg/ml)	pH	Time (hr)	Substrate split (mg/ml)	
					No activator	0.001 M MnSO ₄
Not dialyzed	DL-Leucylglycine	9.4	7.9	2	0.56	2.73
Once	DL-Leucylglycine	9.4	7.9	2	0.38	—
Twice	DL-Leucylglycine	9.4	7.9	2	0.11	0.30
Not dialyzed	DL-Leucylglycylglycine	24.5	7.9	2	1.52	3.13
Once	DL-Leucylglycylglycine	24.5	7.9	2	0.74	—
Twice	DL-Leucylglycylglycine	24.5	7.9	2	0.76	0.76

Table 7 *Dermoprotease activity of Fruton type extracts of fresh rat skin compared with that of dried skin extracts*

(Extracts (5 ml) made as previously described, mixed in each case with 5 ml of a 1% casein solution, and digestion allowed to proceed at 38° for 1 hr. Amino N determined by Van Slyke method.)

Extract	Skin preparation	Amino N (mg/10 ml)		
		Before digestion	After digestion	Increase
Dried skin	—	—	—	0.329*
Fresh skin	1	0.844	0.927	0.083
Fresh skin	2	0.970	1.181	0.211
Fresh skin	3	0.463	0.603	0.139
Fresh skin	4	0.523	—	0.154
Extract of residual skin	4	0.262	—	0.254†

* Average of ten determinations

† One determination

In addition, the skin remaining after the Fruton-type extract had been made was re-extracted with 6% KNO_3 in the proportion 1 g fresh skin (≈ 0.3 g dried skin) to 5 ml 6% KNO_3 and the peptidase and proteinase activities compared with the original extract. Tables 7 and 8 show that there was little peptidase, but good proteinase activity in the final KNO_3 extract.

In one experiment using DL leucylglycine as substrate (9.4 mg/ml) at pH 7.9 and a digestion period of 2 hr, an extract of rat skin gave a splitting of 1.12 mg/ml without addition, which was increased to 1.5 mg/ml by addition of 0.01M- MgSO_4 and to 2.17 mg/ml by 0.002M CoCl_2 . Berger & Johnson (1939) stated that the leucylpeptidase activities of their preparations were unaffected and slightly

Table 8 Comparison of peptidase activities of Fruton type extract of rat skin, and of extract made of residual skin with 6% KNO_3

(Extracts made as described in Table 1)

Extract	Substrate	Concentration of L-substrate (mg/ml)	pH	Time (hr)	Percentage hydrolysis	
					No activator	0.001M MnSO_4
Fruton type	DL-Leucylglycylglycine	12.25	7.9	2	17.6	30.1
Fruton type	L-Leucylglycine	9.4	7.9	2	8.4	5.1
Residual skin	DL-Leucylglycylglycine	12.25	7.9	2	3.4	3.6
Residual skin	DL-Leucylglycine	9.4	7.9	2	2.5	3.1

(2) Some further details about the enzymes

Substrates Precise data about the optimal concentration of substrates could not be found in the literature. The results of varying the amount of leucylglycylglycine are given in Fig. 1, these show that the concentration used by Fruton (1946) is approximately maximal.

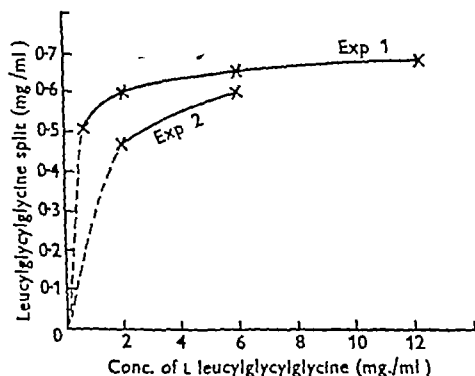


Fig. 1 Effect of substrate concentration pH 7.9, 38°. Acetone dried skin extracted with 2% NaCl. Extract mixed with equal volume of DL-leucylglycylglycine solution and incubated for 2 hr. Estimations by titration.

Metals The results of similar experiments on the variation of activity with varying concentration of manganese are shown in Table 9, and indicate an optimal concentration of 0.002M Mn , it is to be noted that Fruton used 0.001M and Berger & Johnson (1939) found that the leucylpeptidase activity of their extracts of hog intestinal mucosa increased steadily as the concentration of Mn increased from 10^{-6} to 10^{-2} M.

inhibited by cobalt, whereas Maschmann (1940) found that the leucylpeptidase activities of sera were activated by cobalt though less so than by manganese, a result consistent with our experiment.

Table 9 Effect of manganese concentration on leucylglycine activity

(9.4 mg DL-leucylglycine/ml., 2 hr incubation, pH 7.9)

Exp no	MnSO_4 (M)	Leucylglycine split (mg/ml)
1	Nil	0.37
	0.0002	0.80
	0.0006	1.19
	0.001	1.33
	0.002	1.45
	0.005	1.35
2	Nil	1.18
	0.001	4.02
	0.002	4.17

(Experiments 1 and 2 were made with different skin extracts)

DISCUSSION

Comparison of these results upon rat skin with those of Fruton (1946) upon rabbit skin show that the skins differ in the proportions present of the two peptidase activities investigated.

Since the Fruton type extracts contain the skin proteomase, though in weak concentration, it can be concluded from Fruton's negative results with carbobenzyloxy L-glutamyl L-tyrosine and carbobenzyloxyglycyl L-phenylalanine, that the skin proteomase differs from pepsin and carboxypeptidase, and therefore from any of the hitherto recognized groups of proteolytic enzymes. Since, also, it has

recently been shown (Peters & Wakelin, 1948, Grob, 1946) that trypsin and chymotrypsin are inactivated by thiol compounds, whereas the skin proteinase is not, the conclusion stands firm at present that it is a proteinase of different type

SUMMARY

1 The proteinase present in acetone dried extracts and in Fruton type extracts of fresh skin from the rat have been compared and further characterized in the light of Fruton's results

2 The acetone dried extracts have high pro-

teinase activity and low dermo- and amino peptidase activity with only slight activation effects of manganese, whereas the fresh skin extracts have a high peptidase and low proteinase activity

3 The skin proteinase differs from previously recognized enzymes

4 Manganese activates the leucine aminopeptidase optimally at 0.002M, cobalt and magnesium also activate

We are indebted to Prof K Linderström Lang for his hospitality and kindness in showing his technique, also to the Nuffield Committee and to the Osler Trustees (Oxford) for grants in aid of this work

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Separation and Estimation of Saturated C₂-C₈ Fatty Acids by Buffered Partition Columns*

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The lack of adequate small scale methods for the analysis of mixtures of fatty acids has long been felt. Following up an observation of Lester Smith (1942), Elsdén (1946a) has recently introduced an important new technique, based on partition chromatography, for the separation and estimation of the lower members of the saturated series of fatty acids. A wet silica gel was impregnated with bromocresol green, a small sample of the mixture of acids, dissolved in chloroform, was introduced at the top of the column and, after its penetration into the gel had taken place, a suitable mixture of butanol and chloroform was allowed to percolate through the column. By using the colour change of the indicator as a pointer to the positions occupied by the acids, Elsdén was able to estimate propionic and butyric acids. Under certain conditions valeric acid also could be estimated, but the precise behaviour of the gel is conditional upon its properties. Acetic acid could not be estimated except by difference. The method has been used by Elsdén (1946b) and by

Elsden, Hitchcock, Marshall & Phillipson (1946) with excellent results in the analysis of rumen contents, but it has certain disadvantages. Different batches of silica prepared in the same manner often differ considerably in their properties, and of several batches only one may be suitable for use. The range of the method is very limited: acetic acid can only be estimated by difference and the method cannot ordinarily be applied to acids higher than butyric, such higher acids fail to undergo separation and can only be determined collectively. Finally, no separation is possible between the isomers of any given acid.

We have now devised a procedure which obviates most of the disadvantages of Elsdén's method and are able to identify, separate and estimate most of the naturally occurring, steam-volatile members of the saturated fatty acid series with a high order of accuracy and recovery. The use of indicators on the column is dispensed with, acetic acid can be determined directly, small differences in the properties of the silica are of no consequence, and almost complete resolution of certain isomeric valeric acids has been achieved.

* A preliminary account of this work was communicated by one of us (R S) to the Biochemical Society on 31 October 1947 (Scarlsbrick, Baldwin & Moyle, 1948).

In order to extend the range of the method it was necessary to find conditions which would increase the relative solubility of the higher acids in the stationary, aqueous phase. We tried the use of alkaline columns with the idea of altering the effective partition coefficient in favour of the aqueous phase, and even in our earliest experiments we were able to achieve virtually complete separations of caproic and valeric acids (Fig 1). It was

Throughout our experiments we have used columns heavily buffered with phosphate. Neither this nor the employment of alkaline gels is entirely novel (see Syngé, 1946, for further references) but, so far as we are aware, columns of the type used here have not been previously used for the same purpose, although Sato, Bary & Craig (1947) have used buffers in a method based on 'counter current distribution' which allows the separation of fatty acids as far as valeric. Goodall & Levi (1946) have also used buffered chromatograms in a micromethod for the separation of the various types of penicillins on filter paper.

EXPERIMENTAL

Reagents

Solvents Chloroform, previously washed with water and dried over CaCl_2 , and *n*-butanol are redistilled in an all-glass apparatus. Normally we use mixtures containing 1, 10 and 30% (v/v) of *n*-butanol in chloroform (referred to in the text as '1% butanol', etc), all of which are equilibrated by shaking with 2M K_2HPO_4 . It is advisable to filter the equilibrated solvents through dry paper before use.

Silica Silica may be prepared according to the directions given by Gordon, Martin & Syngé (1943) or Isherwood (1946). Several different preparations were used in the course of these experiments but, as we shall show, there is little if anything to choose between different samples.

Buffers Stock 2M solutions of KH_2PO_4 , K_2HPO_4 and K_3PO_4 are required and should be made up from the purest specimens available. We found that a commercial sample of K_3PO_4 gave very poor recoveries compared with a specimen made by adding the theoretical amount of A.R. KOH to A.R. KH_2PO_4 . From these stocks the following standard buffers are prepared: I, 2 vol K_2HPO_4 + 1 vol KH_2PO_4 ; II, 2.5 vol K_2HPO_4 + 3.5 vol K_3PO_4 ; III, K_3PO_4 alone; IV, KH_2PO_4 alone. These are referred to as 'buffers I, II', etc.

General procedure

Columns Five g silica are intimately mixed with 3 ml of the appropriate buffer by grinding in a small mortar. The mass is suspended in about 40 ml. 1% butanol and poured through a funnel into a tube of the usual design (a drawing of the apparatus actually employed is reproduced in Fig 2), and the tube refilled with 1% butanol by means of a separating funnel with a bent outlet (Fig 2A). The solvent is then allowed to percolate through the column for 20–30 min to permit proper packing of the gel and the formation of a firm surface.

Introduction of acids The acids to be analyzed are introduced in the form of a solution, usually in 1% butanol. Samples as large as 50 ml. may be taken if necessary, but for most purposes 1–5 ml. is convenient. When small samples are to be introduced the device illustrated in Fig 2B is useful: the delivery tube is placed in position with its tip a few millimetres above the surface of the gel. As soon as the last of the solvent has drained into the gel the sample is cautiously run in through the delivery tube. At the same moment the tap (T) is closed and the automatic siphon (S) placed in position with a sampling tube

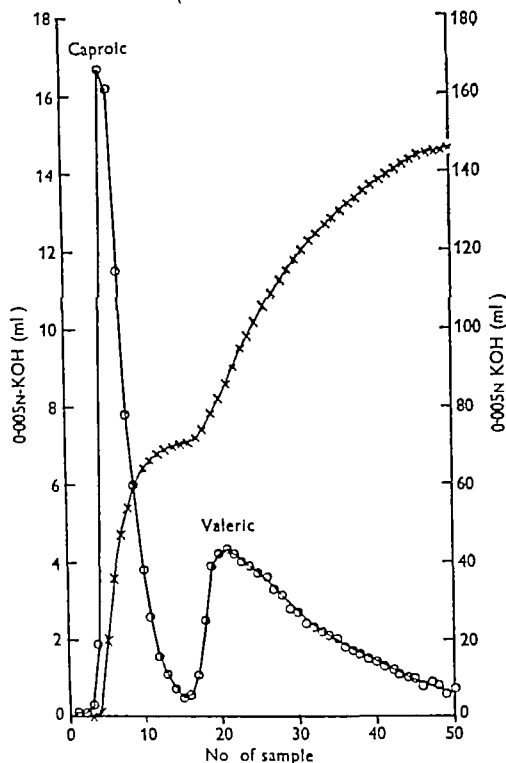


Fig 1 Separation of caproic and valeric acids. Column 5 g silica, 0.5 ml. litmus, 2.5 ml. 2.5M K_2HPO_4 , 0.34 ml. 10N KOH. Solvent chloroform. Ordinates titres of successive 5 ml samples of chloroform eluates, o in individual (scale on left), x aggregate (scale on right).

hoped that by means of even more alkaline columns still higher acids would prove to be separable but, unfortunately, as the length of the fatty chain is increased, the solubility of the soaps decreases rapidly and sets an eventual upper limit to the range of the method, lauric (C_{12}) and higher acids cause blockage of the columns. It is necessary, therefore, to effect a preliminary separation of the lower acids from lauric acid and higher members of the series, this can be done satisfactorily by steam distillation by the method of Friedemann (1938) whereby formic acid, if present, is destroyed. The free, steam volatile acids are then recovered in solution in a suitable mixture of chloroform and butanol by a procedure similar to that of Elsdon (1946a, b).

below The tap (*T*) is then reopened As soon as the sample has drained into the gel the delivery tube is washed down twice with about 1 ml 1% butanol and then withdrawn The separating funnel, already charged with 1% butanol, is placed in position As soon as the washings have entered the gel the tap of the funnel is carefully opened and the solvent admitted to the column

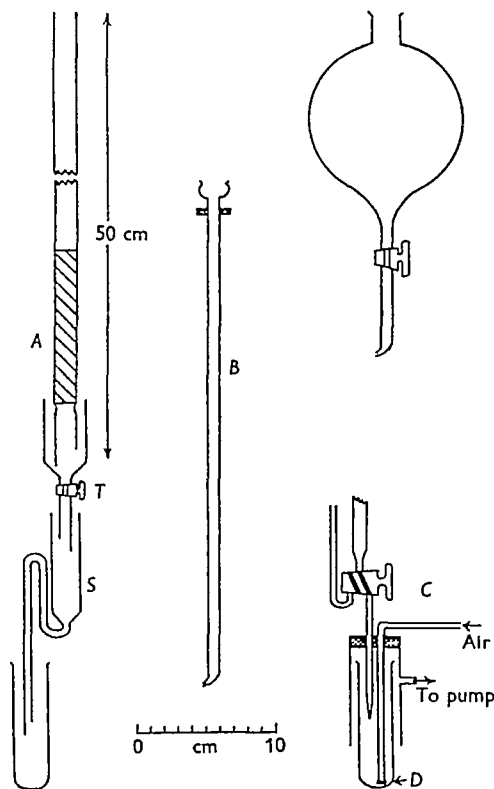


Fig 2 Apparatus (see text)

Sampling Successive, approximately equal samples of about 5 ml (the exact volume is immaterial) are collected in dry tubes (4 in \times 1 in.) by means of the automatic siphon (*S*) Each sample is titrated in turn and the titres, after correction for blanks, are plotted against the number of samples It is usually convenient to plot the titres both individually and cumulatively

Titration To avoid the inconvenience of titrating in a heterogeneous medium 0.005N KOH is used in CO₂ free methanolic solution The solution is stored in a bottle guarded against CO₂ and delivered by gravity to a guarded, self filling 10 ml micro burette, the stopcock of which is lubricated with a silicone preparation

A stream of dry, CO₂ free air is passed through the sample throughout the titration, using a small sintered glass distributor (*D*) to obtain a stream of fine bubbles The air should be passed for at least 30 sec before beginning to titrate, shorter periods of preliminary gassing fail to remove CO₂ while long periods may lead to loss of acids by volatilization The butanol, chloroform and methanol vapours are sucked away by the pump (see Fig 2C)

Indicator and end point In our early experiments we used phenolphthalein as indicator, as is usually advised,

but we later abandoned this in favour of cresol red A 0.04% solution in methanol is used, two drops being taken for each 5 ml solution undergoing titration The colour changes from yellow through a brownish red and finally, very sharply indeed, to a bluish red even when 0.005N alkali is being used, 0.01 ml suffices to produce the final change of colour, which is more easily seen by daylight than by artificial illumination

Cresol red has certain disadvantages which, however, can easily be overcome, and are compensated for by the sharpness of the end point It is preferentially soluble in water so that the samples for titration must be collected in dry tubes, and the air stream must be dried There is a slight lag in the establishment of the final end point, especially in 30% butanol, but this can be overcome by waiting for 30 sec after the end point has apparently been reached in order to see whether the brownish red colour returns There is a further and more subtle difficulty If, when the total titre in 1% butanol is less than about 0.5 ml, a dropwise titration is carried out, a false end point may be reached with the addition of a few drops of the alkali On the addition of a further 1-2 drops the indicator again turns yellow, and only then begins to approach the true end point This curious phenomenon, which is not observable in 10 or 30% butanol, can be avoided by the addition of a few drops of methanol before starting to titrate samples in which a small titre is expected

Blanks Blank values are determined by running samples of the solvents through virgin columns They vary slightly from one sample of silica to another, but the errors introduced by these variations into the final estimates are of the order of not more than 1% when total titres of 10 ml or more are being handled In our own experiments the following average blanks were found

Buffer	Blank (ml 0.005N-KOH/5 ml sample)		
	1 %	10 %	30 %
I	0.023	0.034	0.036
II	0.024	0.032	0.038
III	0.022	0.034	0.036

Change of solvent When one acid has been eluted from the column the elution of the next can be hastened by replacing the solvent by another containing more butanol Generally speaking, the exchange is made when the titre has dropped to the blank level or as soon as an increase of at least 0.1 ml in titre is observed.

General operation It is usually convenient to collect samples every 4-5 min. The rate of flow diminishes with increasing concentrations of butanol and with increasing pH, but depends mainly upon the porosity of the particular sample of silica in use It can be controlled by modifying the proportions of silica and buffer in the mixture, but it is better to rely upon alterations in the hydrostatic pressure of solvent above the column. It is also possible to mix samples of different porosities together in order to obtain suitable rates of flow

An experimental run can be interrupted, if necessary, by fitting to the top of the tube a rubber bung pierced by a small stopcock The tube is filled very nearly to the top with solvent, the bung placed securely in position and the tap closed, in this way it is possible safely to discontinue operations for several hours

Table 1 Behaviour of aliphatic acids (C_2 and upwards) on columns I, II and III

Column	pH*	% butanol	Acids		
			Eluted	By-passed†	Retained
I	6.6	1	C_4	C_2 and higher	—
		10	C_3		
		30	C_2		
II	8.4	1	C_6	C_7 and higher	C_3, C_2
		10	C_5		
		30	C_4		
III	9.5	1	C_7 and higher	—	C_4 and lower
		10	C_6		
		30	C_5		

* Determined by glass electrode (16°) on mixtures of 5 g silica with 3 ml. buffer suspended in 10 ml. water these figures are more useful as a check on the properties of the silica than as indications of the operative pH of the columns

† In the first five to six samples

Operational columns It is not possible to carry out complete separations of more than three acids upon any one column under the conditions we employ, and we have therefore developed three types of columns buffered, respectively, with buffers I, II and III. These columns, referred to as 'columns I, II and III', cover the whole useful range of the method; their performances are summarized in Table 1 (see also Figs 3-5).

RESULTS

Separation and recovery of acids

Recovery experiments were first carried out on carefully redistilled specimens of single acids, the total load taken being of the order of 20 ml. 0.005N in each case. The recoveries are listed in Table 2. Feeling satisfied that virtually quantitative recoveries of single acids can be achieved, we prepared a series of mixtures of known acids and put them through the usual procedure. The results (Table 3, Figs 3-5) showed that virtually complete separations of naturally occurring acids are possible up to C_8 , and that quantitative recoveries of the separated components can be achieved. Oenanthic acid (C_7) is separable from caproic (C_6) but not from caprylic acid (C_8), pelargonic acid (C_9) is inseparable from caprylic (C_8) and higher acids.

Behaviour of isomers *n* and *iso* Butyric acids behave so similarly on the columns that they are indistinguishable and the same is true of *n*- and *isocaproic* acids. We made a special study of the valeric acids, viz *n* and *isovaleric*, trimethylacetic and DL methylethylacetic acids.

Since sharper separations are normally found between acids that come late through the columns (see Figs 3-5) we studied the behaviour of the isomers on columns of type III, from which they are eluted only by 30% butanol. Each experiment was performed on a separate column and all the columns were prepared in the same way and under identical conditions. It was found that by increasing the relative proportion of buffer to silica better

separations were obtained these columns were therefore made from 4 g of silica and 3 ml of buffer III. The results showed that *n* valeric and trimethylacetic acids behave almost identically, while *isovaleric* and DL methylethylacetic acids come through together but considerably later than the other two. We prepared an equimolecular mixture of the *n* and *iso* acids and examined this on one of these columns. The resulting curve (Fig. 6A) showed two well-separated peaks, indicating that a partial separation of the two isomers had been accomplished. Similar mixtures of trimethylacetic and DL methylethylacetic (Fig. 6B), *n* valeric and trimethylacetic (Fig. 6C) and *isovaleric* and trimethylacetic acids (Fig. 6D) were similarly treated and again gave partial separations.

Although we have not succeeded in accomplishing total separations even in these favourable cases, it is, nevertheless, established that *n* valeric and trimethylacetic acids can easily be distinguished from *isovaleric* and DL methylethylacetic acids.

Influence of some experimental conditions

Properties of the silica Several different samples of silica were used in the course of our work. These included one too acid and another too alkaline for use in Elsdon's (1946a) procedure, two more that gave good results by his method, and a sample of Isherwood's (1946) non adsorbent silica. Virtually identical results were obtained with all samples.

Fig. 7 presents the results obtained by analyzing samples of the same mixture of acids (*n* caproic, *isovaleric* and *n* butyric acids) on four different silicas. In examining these curves the following facts must be borne in mind. The precise position taken up by a given acid is influenced to some extent by the rate of flow of the column and therefore by the porosity of the gel. This is shown by slight differences in the positions of the first bands in Fig. 7. More important, however, are the differences due to the fact that the butanol content of the

Table 2 *Recovery of single acids*

No of C atoms	Acid	Column	Amount taken (ml 0.005N)	Amount recovered (ml 0.005N)	Recovered (%)
2	Acetic	I	20.6	20.6	100
3	Propionic	I	18.8	18.2	97
	Propionic	I	18.8	18.4	98
4	<i>n</i> -Butyric	I	19.6	19.4	99
	<i>n</i> -Butyric	II	19.3	18.8	98
	<i>iso</i> -Butyric	I	21.5	21.4	99
	<i>iso</i> -Butyric	II	21.5	20.7	96
5	<i>n</i> -Valeric	II	18.5	18.3	99
	<i>n</i> -Valeric	II	18.5	17.7	96
	<i>iso</i> -Valeric	II	19.0	20.0	102
	<i>iso</i> -Valeric	III	19.0	19.2	98
	Trimethylacetic	II	19.9	20.6	103
	Trimethylacetic	III	19.9	19.7	99
6	<i>n</i> -Caproic	II	17.3	17.3	100
	<i>n</i> -Caproic	III	19.3	19.1	99
	<i>iso</i> -Caproic	III	19.2	19.3	101
7	<i>n</i> -Oenanthic	III	18.8	18.3	98
8	<i>n</i> -Caprylic	III	19.3	19.2	99
9	<i>n</i> -Pelargonic	III	19.2	19.2	100
10	<i>n</i> -Capric	III	18.6	18.5	99

Table 3 *Recovery of acids from mixtures*

Column	Acids taken	Amount taken (ml 0.005N)	Amount found (ml 0.005N)	Recovered (%)	Total recovered (%)
I	<i>n</i> -Butyric	19.6	20.0	102	101
	Propionic	18.8	17.9	96	
	Acetic	20.6	21.4	104	
II	<i>n</i> -Caproic	17.8	17.8	100	99
	<i>n</i> -Valeric	18.5	18.6	100	
	<i>n</i> -Butyric	19.3	18.6	97	
II	<i>n</i> -Caproic	17.8	18.0	101	98
	<i>n</i> -Valeric	18.5	18.6	100	
	<i>n</i> -Butyric	19.3	18.9	98	
II	<i>n</i> -Caproic	17.8	18.6	104	101
	<i>n</i> -Valeric	18.5	18.9	102	
	<i>n</i> -Butyric	19.3	18.8	98	
III	<i>n</i> -Capric	18.6	37.7	100	99
	<i>n</i> -Caprylic	19.3		99	
	<i>n</i> -Caproic	19.3	19.0	99	
III	<i>n</i> -Capric	18.6	37.8	100	99
	<i>n</i> -Caprylic	19.3		99	
	<i>n</i> -Caproic	19.3	18.8	98	
III	<i>n</i> -Capric	18.6	37.5	99	100
	<i>n</i> -Caprylic	19.3		99	
	<i>n</i> -Caproic	19.3	19.4	100	

developing solvent was not increased at the same time in all cases. Such an increase results in a sharp rise in titre as the next acid comes through, and any delay in changing the solvent is naturally followed by a later rise in the curve. When allowances are made for these factors, the differences between the four curves become insignificant, indicating that it is unnecessary to take elaborate precautions in preparing the silica and, incidentally, that different

samples of silica can safely be mixed together in order to obtain columns running at convenient speeds.

Effect of concentration of buffer. We adopted the use of 2M buffers as standard procedure. Fig. 8 shows the effects of using more dilute buffer on columns of type II. Using M instead of the usual 2M phosphates the separation between the first two acids (*n*-caproic and *iso*-valeric) is no longer com-

plete, though the separation between the second pair (*isovaleric* and *n* butyric) is still very good. In 0.5M buffer the second separation is also impaired. Essentially similar results were obtained with the other columns.

sensitive to loads of 0.01 mmol or less, so that small amounts of one acid can be detected and estimated in the presence of larger amounts of others. Excellent separations can be obtained with loads up to about 1 mmol of each acid by the use of columns

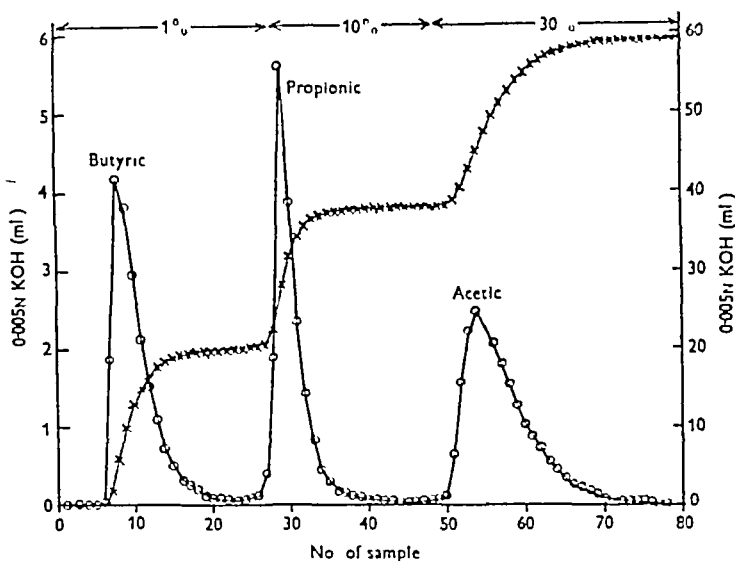


Fig 3 Separation of *n* butyric, propionic and acetic acids (column I). The butanol content of the developing solvent was changed at the points indicated. Acids approx 1 ml 0.1N of each acid. Ordinates titres of successive samples, o individual, x aggregate

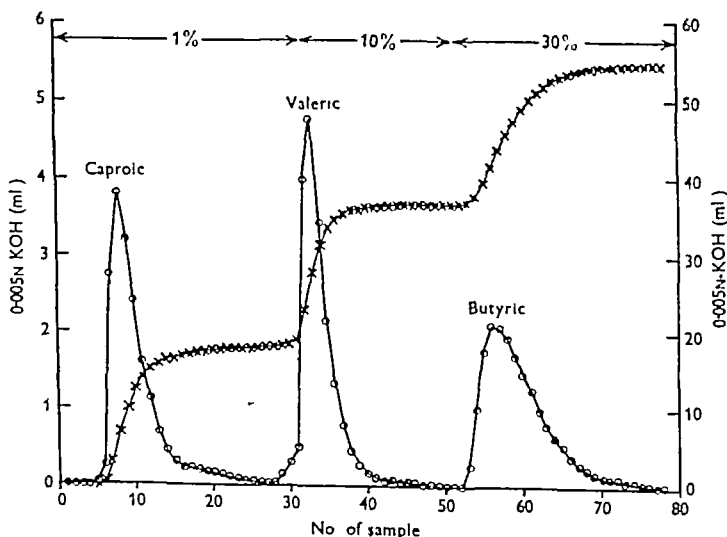


Fig 4 Separation of *n* caproic, *n* valeric and *n* butyric acids (column II). Details as for Fig 3

Effect of load The optimal load for the columns is about 0.1 mmol ($\equiv 20$ ml 0.005N) of each acid in the mixture, corresponding to about 6 mg acetic or 10 mg valeric acid. The method is, however, still

of similar length, but about five times as large a cross sectional area.

Volume of sample. In the method of Elsdén (1946a) it is necessary to introduce the acids to be analyzed

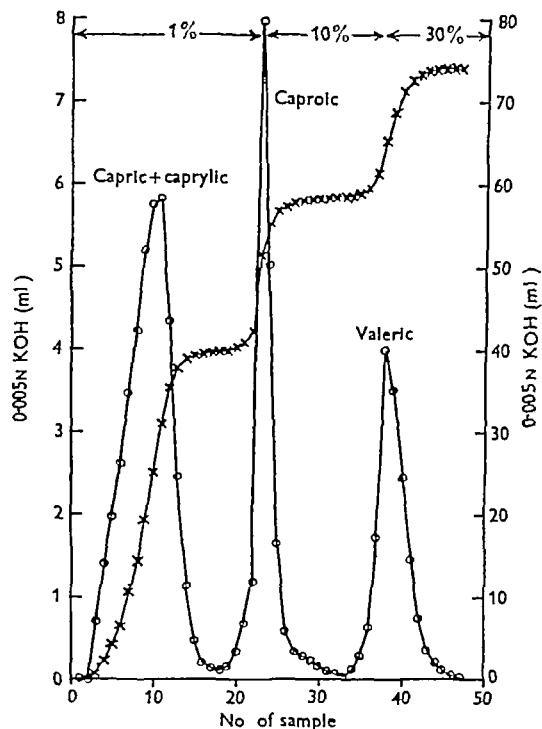


Fig 5 Separation of *n* capric + *n*-caprylic, *n* caproic and *n* valeric acids (column III) Details as for Fig 3

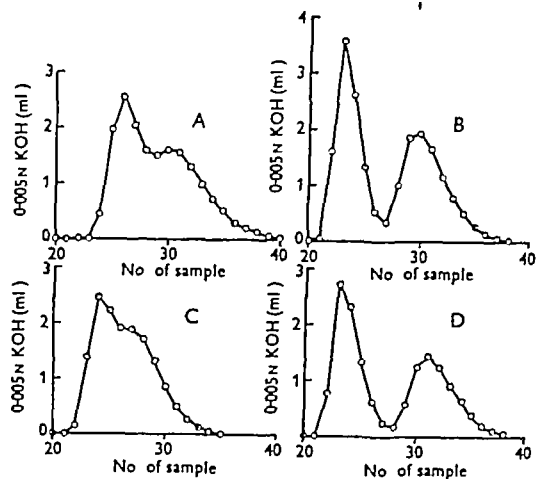


Fig 6 Behaviour of isomeric valeric acids (column III)
A, 0.5 ml 0.1N *n* valeric + 0.5 ml 0.1N isovaleric acids
B, 0.5 ml 0.1N trimethylacetic + 0.5 ml 0.1N-DL-methyl ethylacetic acids
C, 0.5 ml 0.1N *n* valeric + 0.5 ml 0.1N trimethylacetic acids
D, 0.5 ml 0.1N trimethyl acetic + 0.5 ml 0.1N isovaleric acids Solvent 30% butanol in chloroform throughout

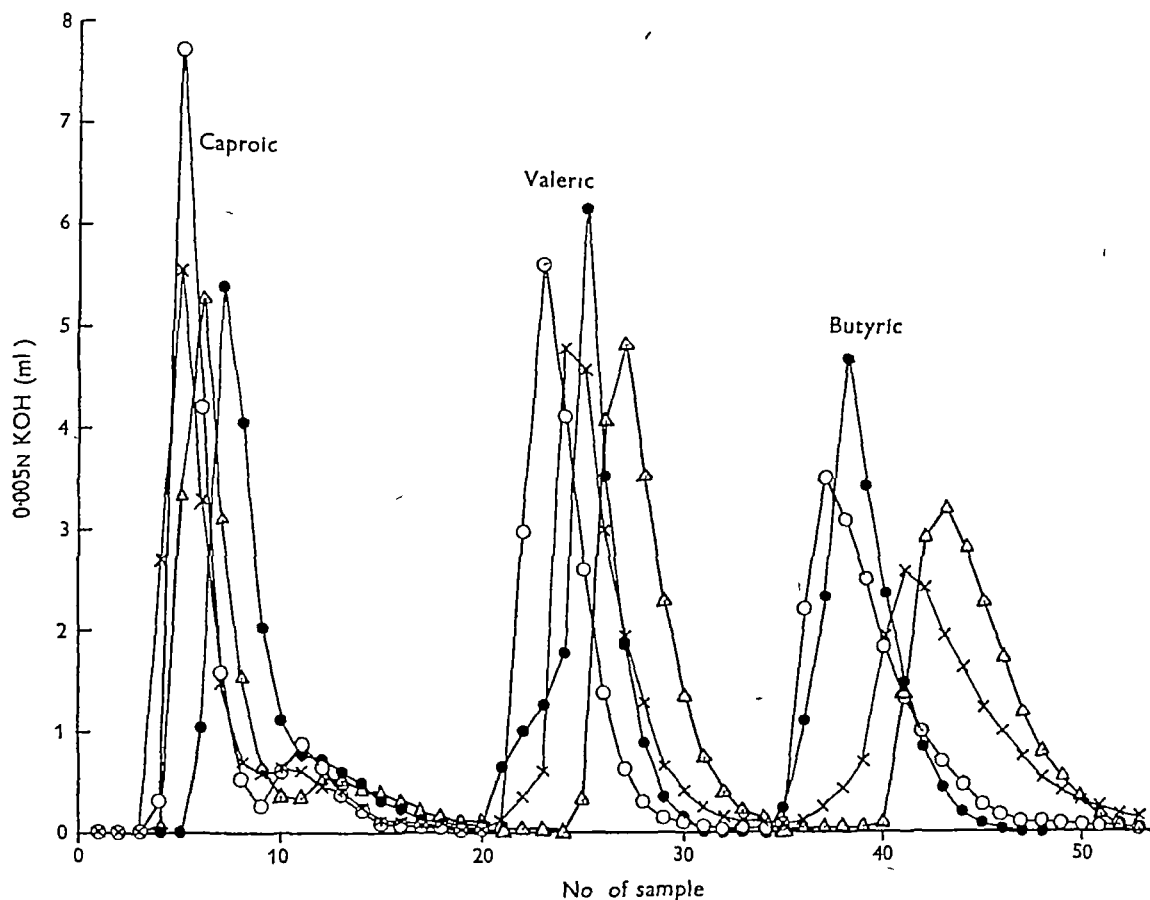


Fig 7 Analysis of a mixture of *n*-caproic, isovaleric and *n*-butyric acids (column II) Four equal samples of the same mixture were separately analyzed on four different samples of silica Note the solvents were not changed at the same point in each case Acids approx. 1 ml 0.1N of each acid

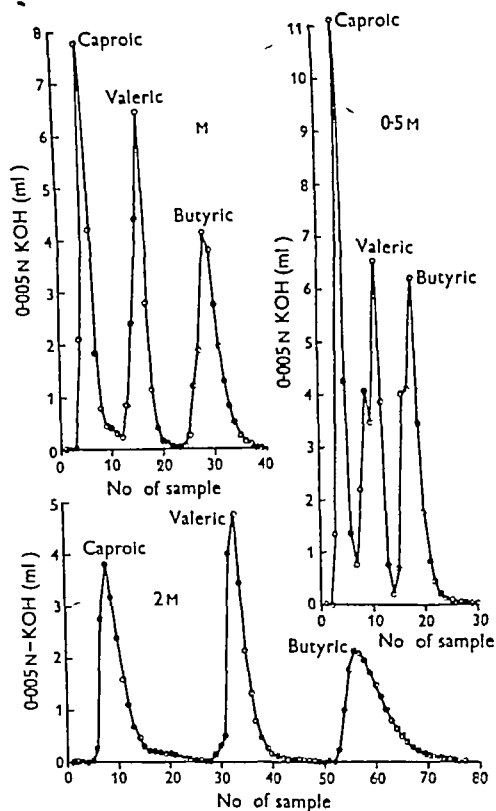


Fig 8 Influence of concentration of buffer on separation of *n* caproic, isovaleric and *n* butyric acids on column II. Acids 1 ml 0.1N approx of each acid

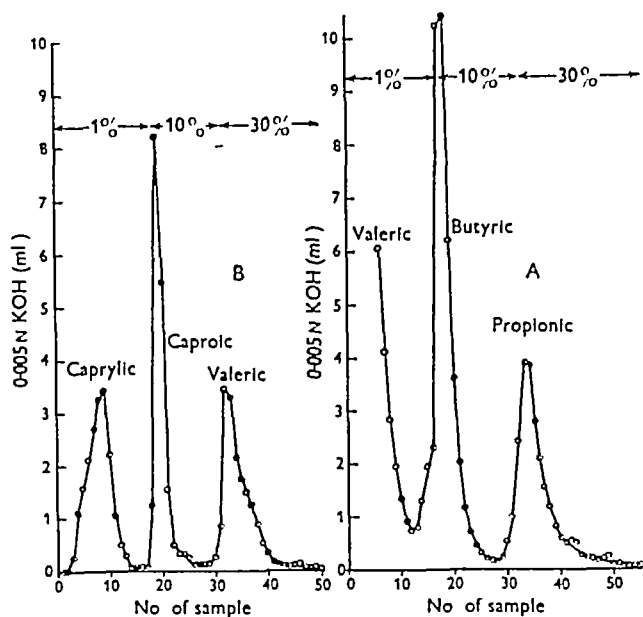


Fig 10 Approximate analysis of a mixture on two columns (see text) Sample, 1 ml A, Column IV, the first five samples were massed and placed on B (column III)

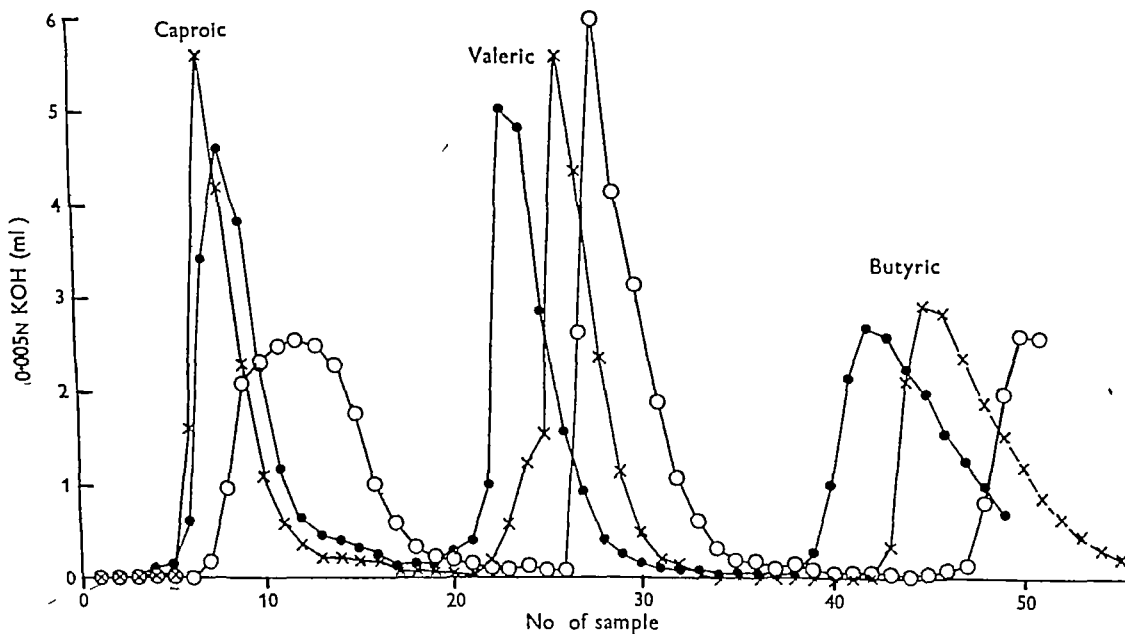


Fig 9 Effect of volume of sample (column II) Separation of *n*-caproic, *n*-valeric and *n*-butyric acids. Acids equivalent of 1 ml 0.1N approx. of each acid. Volume of sample o, 50 ml, •, 20 ml, x, 10 ml.

in a total volume of not more than 3 ml. With the 5 g buffered columns, even when samples of 50 ml are taken, there is still excellent resolution, though the first band is considerably broadened (Fig 9).

Applications of the method

Identification and approximate assay of components of a mixture Each acid shows strongly characteristic behaviour on the columns. Thus the valeric acids are by-passed in the first five to six samples of 1 % butanol by column I, eluted rapidly by 10 % and only very slowly by 1 % butanol from column II, and rapidly eluted only by 30 % butanol from column III. So characteristic is the behaviour of each member of the series of acids studied that it may be taken as evidence of identity, with the reservation that only certain pairs of the isomers we have tested proved to be distinguishable (p 311), even under carefully controlled conditions.

Before proceeding to exact analysis it is often desirable to carry out an approximately quantitative analysis of an unknown mixture in order to discover what acids are present and in what concentrations. This can be done by taking advantage of the large sample volumes that the columns can handle. For this purpose an additional column (IV) is of great service: this is buffered with 2M-KH₂PO₄ alone, and by-passes caproic and higher acids, yields up valeric with 1 %, butyric with 10 % and propionic with 30 % butanol. Acetic acid is retained but can be approximately assayed by difference.

A small, measured sample of the mixture to be analyzed is placed on a column of type IV and the first five samples, which contain some of the valeric acid together with practically the whole of the higher acids, are not titrated but massed together and reserved. The column is then developed in the usual manner. A column of type III is then set up, the five reserved samples are placed upon it and the column then developed. The results of such an experiment are shown in Fig 10: the acids taken in this case consisted of a 1 ml sample containing about 0.1 mmol each of acetic, propionic, *n*- and isobutyric, *n*- and isovaleric, *n* caproic and *n*-caprylic acids.

Only approximate results can usually be obtained in this manner, but the procedure gives reliable indications of the nature of the acids present and approximate values for their respective concentrations. It is then possible to select columns suitable for exact analysis under optimal conditions.

Routine analyses of mixtures Where routine analyses are to be carried out, and the identities of the acids present have been established, it may not be necessary to employ all three of the standard columns. In the analysis of rumen contents, for example, in which only acetic, propionic and butyric

acids are usually assayed (Elsden, 1946b, Elsdén *et al* 1946, for example), only column I will be required. In a hypothetical case where, say, only valeric, butyric and propionic acids were to be determined, a column intermediate between types I and II could be specially designed for the purpose. It is reasonable to assume that intermediate columns would yield results as quantitative as those obtained on the standard columns used in the present experiments. In addition, higher acids by-passed by one column might be put directly on another that is more alkaline.

Detection and estimation of impurities: purification of acids As the method is sensitive even to very small loads, it is not difficult to detect the presence of traces of contaminants in supposedly pure specimens of acids. We were able, for example, to demonstrate the presence of approximately 4 % of a valeric and 6 % of propionic acid in a redistilled sample of 'pure' isobutyric acid. In another experiment an allegedly pure specimen of *n* caproic acid was found to contain no less than 17 % of an unknown impurity.

Since the columns can handle loads up to about 0.2 mmol of acid, specimens of this order can be freed from traces of impurities by passage through an appropriate column and subsequent recovery by neutralization and evaporation to dryness.

Preparation of characteristic derivatives Considerable amounts of purified acids can be obtained by the procedure outlined in the preceding paragraph and are of great value for the preparation of characteristic derivatives, such as the piperazonium salts or phenylhydrazides, for purposes of identification. The main difficulty likely to be encountered here lies in the removal of the indicator, but we avoid this by proceeding in the following manner.

A suitable column is set up and loaded with about 0.2 mmol of each of the acids to be characterized. Samples are collected and titrated in the usual manner until the first acid begins to come through. The next six samples, which contain the bulk of this acid, are massed together, without titration, for subsequent recovery. Further samples are then titrated until the second acid begins to come through and this is likewise collected in six samples, again without titration, and so on. When more than 0.2 mmol of an acid is required it is an easy matter to run more than one column and mass the corresponding eluates or to work with wider columns.

Limitations of the method

As the foregoing discussion indicates, this new method possesses numerous advantages over its predecessors. Certain drawbacks remain, however, in particular the inability of the columns completely to resolve mixtures of isomeric acids and of acids higher than caprylic.

It seems to be well established (Hilditch, 1947) that, with the exceptions of propionic and isovaleric acids, fatty acids containing either a branched chain or an odd number of carbon atoms occur but rarely in nature. Our procedure is therefore capable of separating and analyzing all the naturally occurring steam volatile saturated fatty acids as far as C_8 . We have shown that oenanthic (C_7) acid can be completely separated from caproic acid (C_6) but not from caprylic (C_8), while pelargonic (C_9) is inseparable from caprylic (C_8) and capric (C_{10}). While for biochemical purposes this limitation is perhaps not a serious one, for purely chemical work, in which odd numbered acids are more likely to be encountered, the present method will break down in cases where acids containing more than seven carbon atoms have to be dealt with.

SUMMARY

1 A new method is described for the identification, separation and estimation of most of the steam volatile members of the saturated series of fatty acids. The procedure is based on the principle of partition chromatography heavily buffered silica

gels, without indicator, form the stationary phase of the partition columns, the moving phase consisting of mixtures of chloroform and butanol.

2 Naturally occurring fatty acids from acetic to caprylic can be identified, quantitatively separated and estimated. Caprylic cannot, however, be separated from higher acids.

3 Of the non natural acids, oenanthic can be separated from lower but not from higher acids.

4 Partial separation has been achieved between isomeric forms of valeric acid but isomeric acids cannot in general be distinguished or separated.

5 Acids can be recovered from the columns in quantities large enough to permit the preparation of characteristic derivatives.

6 Optimal results are obtained with quantities of the order of 0.1 mmol of each acid, whether taken separately or in mixtures.

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Note. The range of the method has been improved by the use of a column buffered with 9 parts of 2 N KOH to 1 part of a 2 M solution of glycine

and KCl. Caprylic (C_8) and higher acids are eluted with 1 % butanol, oenanthic (C_7) with 10 % butanol and caproic (C_6) with 30 % butanol.

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Experimental Evidence that the Stem-end Blackening Pigment of Potatoes is a Compound of Iron

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In studies of the environmental conditions of growth which lead to the production of potatoes liable to show stem end blackening, the quantity of pigment present was estimated by visual judgement (Wager, 1946, 1947). In the course of experiments to develop an alternative, physical method for the estimation of the stem end blackening pigment, it was found that the intensity of the colour of the extracts was increased by the addition of small amounts of iron salts, and further work was directed to this point.

Robison (1941) suggested that the pigment might be a compound of iron. The evidence given was concerned with the relationship between the iron content of the tubers and the amount of stem end blackening shown by them, and was conflicting. She also suggested that the blue black compound was an oxide of iron. These views were criticized by Cowie (1941). Nutting (1942), on the basis of analogy with other pigments, suggested the possibility that the blue black pigment was a metallic complex, but offered no supporting evidence.

METHODS

The potato extracts were prepared from median longitudinal slices, 3 mm thick, of peeled tubers. 40 or 50 potatoes were sliced to obtain a composite sample. The slices were washed free of surface starch, cooked in glass distilled water, reduced to a sludge and left to stand for about 1 hr. to allow time for equilibration of soluble material between the potato cells and the liquid. The potato cells were then filtered off and the cloudy filtrate left to stand with chloroform for several days, during which time precipitation occurred. One or two successive filtrations gave an extract with only a trace of opalescence.

The stem end blackening pigment is sensitive to change in acidity, being colourless or nearly so at an acidity of low pH (3 or less), and attaining a maximal intensity of colour at one of pH 8-9 (Wager, 1945). This change in colour was the basis of the method of estimating the pigment. The absorption of light by the solution was measured at pH 7.40 and also at about pH 3, and the difference between these two values was used as an estimate of the content of stem end blackening pigment. This estimation was fairly specific, but in some cases there may have been a slight change in the degree of opalescence of the solution on acidification, and it seems probable that in all extracts there were small amounts of compounds which were pale yellow in alkaline solution and colourless in acid solution, such compounds

could be seen in some of the extracts containing little stem-end blackening pigment.

Iron was estimated by $\alpha\alpha'$ -dipyridyl using the potato extract prepared as above (i.e. without ashing). The development of colour was slow, 3-4 hr. were required for maximal intensity, whereas in pure solutions of iron 15 min. only. The average recovery of iron added to the solution was within about 2% of the value shown by the calibration curve, but individual values showed some experimental error. The complete recovery of amounts of iron as low as 1 $\mu\text{g}/\text{ml}$. from solution with 3-4 times this amount of iron, as determined by the $\alpha\alpha'$ -dipyridyl reagent, suggests that all the ionizable iron present was estimated, and this has been assumed.

pH was determined with a glass electrode, and light absorption by a photometer (Hilger Spekker) using a violet filter, Iford no. 601.

The samples of potatoes used in this work were all commercially grown in East Anglia. They came from a variety of soil types and consisted of five samples of King Edward, five of Doon Star and eleven of Majestic potatoes. (The author's thanks are due to Mr B. S. Smith of the Potato and Carrot Division of the Ministry of Food, Peterborough, who arranged for the collection of these samples.)

RESULTS

Extracts prepared from tubers liable to stem-end blackening are grey in colour at pH 6 and become somewhat browner as the pH is adjusted to 7.5. The addition of a solution of an iron salt, which increases the iron concentration by a few $\mu\text{g}/\text{ml}$, leads to a marked increase in intensity of colour. This increase in intensity of colour may be explained by the assumption that the stem end blackening pigment is a compound of a colourless precursor and iron, and that there is normally an excess of the colourless precursor. On the other hand, there might be present two pigments, the stem end blackening pigment and another which was affected by iron, e.g. tannin. To test whether tannin was present an extract with a high intensity of colour was shaken at intervals with droplets of gelatin for 2 hr. The gelatin was filtered off and melted, and then to the diluted and melted gelatin and to the filtrate was added an excess of iron (10 $\mu\text{g}/\text{ml}$). The change in intensity of colour with pH was determined for both solutions. The gelatin contained slightly less material than increased in colour with added iron than did the

filtrate, i.e. there was no evidence of concentration in the gelatin. The same technique when applied to a solution of tannic acid of similar concentration resulted in a marked concentration of tannic acid in the gelatin. As a further test for the presence of tannin an attempt was made to absorb the pigment on goldbeater's skin. This was also negative. There is, therefore, a *prima facie* case for assuming that the increase in colour with iron is not due to the presence of tannins.

Extracts were prepared from different samples of potatoes and their pH was adjusted to 7.40. To 10 ml portions of these extracts 1–15 $\mu\text{g/ml}$ of iron was added as ferrous sulphate. The concentration of iron and also the amount of stem end blackening pigment were then determined, with the results shown in Fig 1. The lowest points on the curves are for the extracts with no added iron.

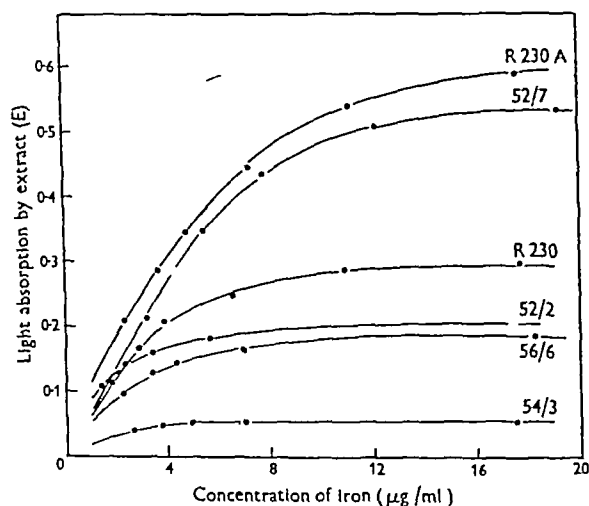


Fig 1 The relationship between the estimated content of iron in potato extracts and the concentration of stem end blackening pigment, expressed as the optical density of the extract

If all these curves, when extrapolated backwards, had passed through the origin it would have been clear evidence that the stem end blackening pigment was a compound of iron. Some of the curves, however, when extrapolated, do not appear to pass through the origin, and it seems reasonable to assume that in these cases there were present compounds, sensitive to the change in pH, other than the stem end blackening pigment. A faint yellow colour at pH 7.4, which was lost in acid solution, was noticed in some extracts which contained very little stem end blackening pigment. The curves in Fig 1, therefore, offer fair evidence for the combination of a colourless precursor with iron to give the stem end blackening pigment.

The colour of any given extract is clearly dependent on the concentration both of iron and of the colourless precursor of the stem end blackening pigment. The maximum intensity of pigment obtained with a high concentration of iron may be taken as a measure of the total amount of stem end blackening pigment present. This has varied in different extracts from 6 to 31 units, whilst the concentration of iron in the extract merely varied from 1.4 to 3.5 $\mu\text{g/ml}$ and could not be correlated with the variation in stem end blackening pigment. It follows, therefore, that the major factor in determining the amount of blackening that develops in potatoes after cooking is the content of the stem end blackening pigment precursor and not the content of iron (cf. Robison, 1941). The relationships between the content of iron, the content of stem end blackening pigment, and the variety and conditions of growth of the tuber will be discussed in a subsequent paper.

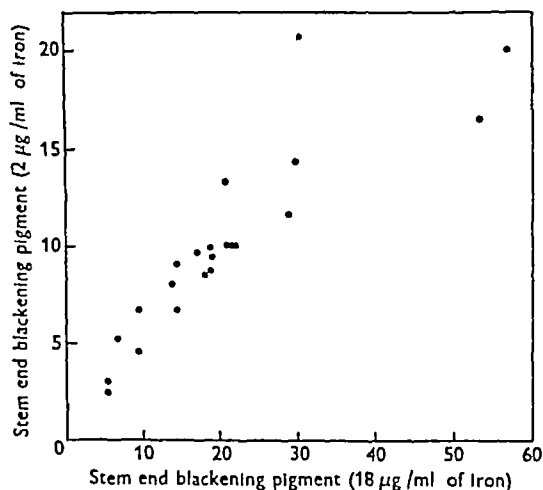


Fig 2 The concentration of stem end blackening pigment, expressed as optical density, in potato extracts at a low concentration of iron, 2 $\mu\text{g/ml}$, plotted against the concentration of pigment in the same extract at a high concentration of iron, 18 $\mu\text{g/ml}$

If these extracts contained coloured stem-end blackening pigment and a different colourless compound, liable to darken with iron, it would be very unlikely that the concentration of both compounds would always be in the same ratio. Unless this were so the intensity of colour developed at a high concentration of iron would bear no constant ratio to that at a low concentration. These two values have been plotted against each other in Fig 2 for all extracts prepared, and a sensibly constant ratio between them is clearly shown. Some small divergencies from a constant ratio must be expected in view of the experimental errors and of the presence of other light absorbing compounds in some of the

extracts The view that the extracts contained only one main compound that combines with iron, and that this combination gives rise to the stem end blackening pigment receives support, therefore, from Fig 2

A simple but very conclusive visual demonstration of the combination of the stem end blackening pigment with iron may be made by immersing one half of cooked tubers, showing stem end blackening, in a solution of 10 μg /ml of iron for about 1 hr and immersing the opposite halves in distilled water as a control The iron causes a great intensification of colour in regions previously blackened, but a

negligible discoloration occurs in the rest of the tuber

SUMMARY

Evidence is offered to support the view that the stem-end blackening pigment of potato is a compound of iron with a colourless precursor The concentrations of iron and of the precursor vary in different samples of potatoes The range of concentration of the precursor is greater than that of iron

The work described above was carried out as part of the programme of the Food Investigation Board of the Department of Scientific and Industrial Research

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THE BIOCHEMICAL SOCIETY

The 268th Meeting of the Society was held in the Biochemistry Department, The University, Glasgow, W 2, on Friday and Saturday, 23 and 24 July 1948, commencing each day at 11 15 a m, when the following papers were read

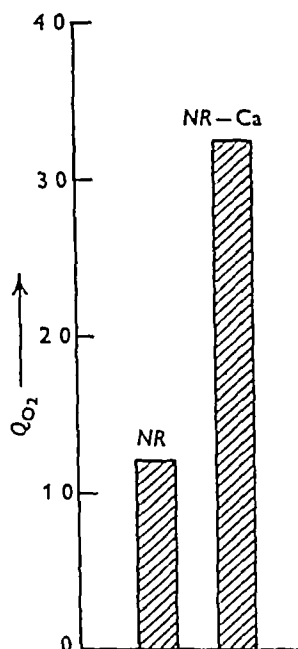
COMMUNICATIONS

Selective Influence of Calcium on the Respiration of Skeletal Muscle By A LASNITZKI (Medical School, University of Birmingham)

It has been found that the rate of respiration of various animal tissues diminished to a more or less degree if calcium was removed from the surrounding medium (Lasnitzki, 1934). The tissues examined included liver, kidney, spleen, brain cortex from the rat, intestinal mucosa from the rabbit, and two types of transmissible rat tumours. Similar effects of calcium deficiency were observed in respect of tissue glycolysis. It is to be noted, further, that the rate of respiration and glycolysis, of normal as well as tumour tissue, was likewise reduced if the medium did not contain potassium. This result prompted the hypothesis that calcium acted in an indirect manner, namely, through inhibition of the outward diffusion of potassium from the cell. The hypothesis was substantiated by the results of investigations on the influence of calcium upon the potassium content of tumour tissue, kept under conditions similar to those maintained in metabolism experiments (Lasnitzki, 1938).

In contrast to these findings, absence of calcium from the medium was found to have a stimulating effect upon the respiration of skeletal muscle, while the reaction to lack of potassium was unaltered. The material employed was the diaphragm of rats of 100–120 g weight. In each experiment about one half of the muscular portion of one diaphragm was suspended in normal (glucose free) Ringer solution having a low bicarbonate content, and the other half in a similar solution in which CaCl_2 (0.0021M) had been replaced by an equimolecular amount of NaCl. Oxygen consumption was measured (37.5°, atmosphere of pure O_2) according to Warburg (1930), tissue pieces were gently shaken in larger volumes of corresponding suspension fluids prior to measurement. The results obtained during the first 30 min observation are illustrated in the accompanying figure, giving the average of three experiments. It will be seen that the rate of respiration in normal Ringer solution (NR) was fairly small, but that it increased more than 2½ times when calcium had

been excluded (NR – Ca). The difference was maintained during subsequent periods of observation, although it became gradually less. Calcium is thus able to exercise a direct inhibitory action upon the respiration of skeletal muscle. No effect of this kind has, so far, been obtained with heart muscle tissue.



The most likely explanation of this selective influence appears to be that whereas calcium may yet cause a limited inhibition of the outward diffusion of potassium from the skeletal muscle fibre, it is capable at the same time of penetrating that cell to a greater extent than the cells of the remaining tissues. In this way calcium will get in a position to counteract, through its dehydrating power, the stimulating action of potassium (cf Lasnitzki, 1945).

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The Measurement of the Cytochrome Oxidase Activity of Tissue Preparations By E C SLATER (Molteno Institute, University of Cambridge)

The cytochrome oxidase activity of tissue preparations is usually measured by determining the rate of oxygen uptake when various reducing agents are added to a mixture of the preparation (in phosphate buffer) and pure cytochrome *c*. It is assumed that the cytochrome *c* is reduced by the reducing agent as soon as it is oxidized by the cytochrome oxidase-oxygen and that the rate of oxygen uptake is dependent only on the cytochrome oxidase activity.

It has been found that this assumption is valid only when enzyme preparations of relatively low cytochrome oxidase activity (e.g. Keilin & Hartree's 1938 preparation) are employed. With the much more active preparations used more recently by Keilin & Hartree (1940, 1947), the rate of oxygen uptake depends not only on the cytochrome oxidase activity, but also on the concentrations of cytochrome *c* and reducing agent and on the catalytic activity of the added cytochrome *c*, which is affected by the concentration of the phosphate buffer. The concentrations of cytochrome *c* required for maximal activity are so great that an indirect

method, involving extrapolation of the measurements at different cytochrome *c* concentrations to infinite cytochrome *c* concentration, must be employed. By this method the Q_{O_2} (fat-free dry weight—38°) of the Keilin & Hartree (1947) preparation is 3400.

p-Phenylene diamine, unlike other reducing agents, is rapidly oxidized by the enzyme preparation in the absence of added cytochrome *c* (Keilin & Hartree, 1938), owing to its ability rapidly to reduce the endogenous cytochrome *c* in the heart muscle preparation. The activity measured at infinite cytochrome *c* concentration is, however, independent of the reducing agent, showing that there is no alternative pathway, in the heart muscle, for the oxidation of *p*-phenylene diamine, as has been suggested by Stotz, Sidwell & Hogness (1938).

Catechol and adrenaline are not satisfactory reducing agents for this determination. The former has too high an oxidation-reduction potential, while adrenaline is rapidly oxidized by cytochrome *c*, even in the absence of cytochrome oxidase.

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A Method of Separating the Main Chemical Constituents of *Pisum sativum* By MARGARET B BROWN, R P COOK and K M STEWART (Department of Biochemistry, University College, Dundee)

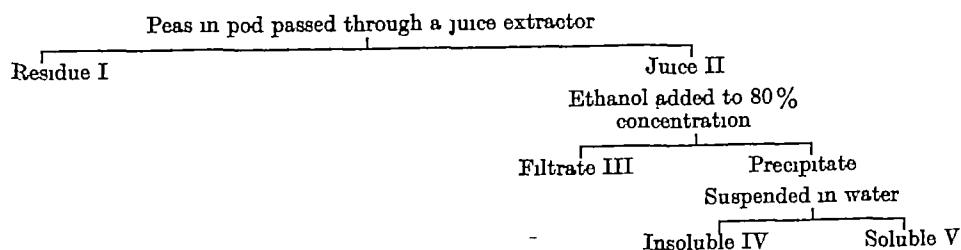
During the course of an investigation on a fraction present in peas which stimulated penicillin formation (Cook, Tulloch, Brown & Brodie, 1945), data were accumulated on the chemical constituents present in the pea. The 'Gladstone' variety of pea has been studied.

The following method of separation was employed

The general chemical composition of these fractions is shown in the table (Values are expressed as g/100 g of dry peas in pod.)

Fraction I consists of fibre (26%), starch (40%) and protein.

Fraction III consists mainly of di- and mono-saccharides (72%, estimated as glucose). From it



have been isolated sucrose, fructose (as the diacetone compound) and asparagine

Fraction IV consists of protein and starch
 Fraction V is the fraction active in stimulating penicillin formation. It contains a complex carbohydrate with glucose, fructose and galactose units, nucleoprotein, peptides and free amino acids. The following amino acids have been detected and estimated in the hydrolyzed material: lysine, histidine, arginine, aspartic acid, glutamic acid, glycine, valine, leucine, threonine, cystine, phenylalanine, tyrosine, tryptophan and proline.

Fraction	Dry matter	Ash	Total nitrogen
I	76.8	3.4	1.64
II	23.2	0.6	1.27
III	12.0	—	0.36
IV	4.2	—	0.36
V	6.4	—	0.45

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Phosphatase of Rabbit Polymorphonuclear Leucocytes By D. M. CRAM and R. J. ROSSITER (Department of Biochemistry, University of Western Ontario, London, Canada)

Using the method of King & Armstrong (1934) it was found that polymorphonuclear leucocytes obtained from the rabbit by the method of de Haan (1918) contain an active phosphatase. That such an enzyme may be present in white cells was suggested by Kay (1930) and confirmed by Roche (1931) and Flessinger & Boyer (1936).

In the preparations used in the present study at least 95% of the cells were polymorphonuclear. The pH activity curve showed two maxima, one in the region of pH 10 and one in the region of pH 5. This was confirmed using a number of buffers. The alkaline phosphatase was much more active than the acid phosphatase. Under the conditions of study, the alkaline phosphatase activity was proportional to the concentration of enzyme. The effect of substrate concentration on the alkaline phosphatase activity has been studied and the Michaelis constant (K_m) determined. An excess of substrate inhibited the enzyme. The time course of the reaction has been plotted and shown to be a straight line for at least 60 min; after 90 min the activity fell off in close approximation to the monomolecular law.

Magnesium and glycine, in low concentrations, caused an increase in activity, the optimum concentration of magnesium being $10^{-3}M$. Zinc, cyanide, borate, phosphate, bile salts and glycine, in higher concentrations, were inhibitory. Fluoride had no demonstrable effect. Surface active substances such as saponin, bile salts or 'alkyl sulphate' liberated the enzyme from the cells.

Similar results were obtained using α -glycerophosphate and β -glycerophosphate as substrates. The enzyme hydrolyzed β -glycerophosphate more readily than α -glycerophosphate. The alkaline phosphatase can be considered to belong to class AI of Folley & Kay (1936) and the acid phosphatase to class AII. The alkaline phosphatase can also be considered as a phosphatase II of Cloetens (1939). It is interesting to note that Gomori (1941) demonstrated the presence of alkaline phosphatase in polymorphonuclear leucocytes histochemically.

* An extremely active preparation provided by the Procter and Gamble Company, Cincinnati, Ohio.

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Lipids of Tissue from Nervous System By A C JOHNSON, A R McNABB and R J ROSSITER
(Department of Biochemistry, University of Western Ontario, London, Canada)

The concentration of free and total cholesterol (method of Schoenheimer & Sperry, 1934), cerebroside (method of Brand & Sperry, 1941), total phospholipid, monoaminophosphatide and lecithin (method of Hack, 1947) was determined in the whole brain of a series of guinea pigs, cats and rabbits. From these figures the concentration of ester cholesterol, sphingomyelin and cephalin was calculated. The same estimations were also done on both the white matter and grey matter from brains of the cat, dog, beaver and man.

On a wet-weight basis, there was a higher concentration of cerebroside, total cholesterol, total phospholipid, lecithin, sphingomyelin and cephalin in white matter than in grey. Referred to total 'essential lipid' the concentration of cerebroside and total cholesterol was higher in white matter than in grey, while the concentration of total phospholipid was higher in grey matter than in white. The concentration of sphingomyelin was, however, greater in white matter than in grey, this

being offset by a greater concentration of both lecithin and cephalin in grey matter than in white.

Similar studies were also done on peripheral nerves from the cat, dog, beaver and man. The distribution of the 'essential lipids', i.e. cerebroside, total cholesterol and total phospholipid, and of the various fractions which go to make up the total phospholipid, i.e. lecithin, sphingomyelin and cephalin, more closely resembled the distribution of the lipids in the white matter of the brain than that of either the grey matter or the brain as a whole. In peripheral nerve, however, there was a greater concentration of sphingomyelin and less cephalin and cerebroside.

Both white matter and peripheral nerve were thus characterized by a relatively high concentration of cerebroside, cholesterol and sphingomyelin. It is tempting to speculate, therefore, that it is these lipids, rather than lecithin and cephalin, that form the basis of 'myelin'.

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The Effect of BAL on the Urinary Copper Excretion of Sheep By JAMES STEWART and HAMISH ROBERTSON (Animal Diseases Research Association, Moredun Institute, Edinburgh)

A preliminary study has been made of the effect of 2,3-dimercaptopropanol on the urinary excretion of copper in individual non-pregnant sheep, with a view to ascertaining whether any significant lowering of (a) blood copper level or (b) the copper reserve in the liver, can be brought about. The value of BAL as an agent in the study of the disease occurring in sheep known as Swayback might thus be assessed.

The experiments were carried out in metabolism cages permitting the collection of bulk urine over 24 hr periods.

The BAL was injected intramuscularly as a 5% (w/v) solution in arachis oil containing 10% benzyl benzoate, and the copper determined by the method of Eden & Green (1940).

In case no. 988, weight 100 lb, the experiment extended over a period of 60 days in order (a) to study the effect of varying doses, (b) to see whether the value for the increase in urinary copper could

be maintained for subsequent doses given at intervals up to 20 days.

The following points of interest were noted.

(1) The effect of a given dose of BAL (5 ml) on the urinary copper excretion varied within wide limits for different sheep (2-15 times normal level).

(2) The increase of copper excretion after BAL injection is due to two factors: (a) excretion of copper in conjugation with the BAL, (b) a secondary effect due to increase in urine volume. This may be prolonged over several days. This increase in volume is extremely variable.

(3) No change in the value for copper in the blood could be found after injection of 4 ml BAL.

(4) In the case of animal no. 988 over the total period of 60 days, for a total of 20 ml BAL injected there was an estimated total loss of copper through the urine of 7 mg in excess of the normal excretion level. It was not possible to check the liver copper

of this sheep at the end of the experiment, but it was calculated that the amount of copper stored in the liver of a comparable sheep would be 10-13 mg

(5) There is no apparent change in faecal copper output

In another series of experiments no significant change has been brought about as yet in the blood-copper value of pregnant ewes nor in the liver copper level of lambs given intensive doses of 2-3 dimercaptopropanol

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The Cytological Interpretation of the Feulgen Reaction By EDGAR STEDMAN and ELLEN STEDMAN (Department of Biochemistry, University of Edinburgh)

Feulgen's reaction for deoxyribosenucleic acid is used extensively in cytological work. When applied to sections or similar preparations containing dividing cells the chromosomes, and these only, become stained a violet colour, a result which is generally interpreted as indicating that the whole of the nucleic acid contained in the nucleus is located in the chromosomes. From the fact that the dye produced in Feulgen's reaction is soluble in water it is, however, clear that only structures in the cell which remain insoluble under the staining technique employed and which are capable of adsorbing or otherwise retaining the soluble dye will become stained by this method. On these and other grounds we (1943, 1947a, b) have contested the above interpretation, but our conclusions have been criticized. In particular, Brachet (1946) states, as evidence against our view, that the isolated nuclei of avian erythrocytes retain the whole of their

nucleic acid during the hydrolytic process. We have therefore examined some of the chemical changes which occur in the isolated nuclei from ox liver cells when submitted to treatment similar to that used in Feulgen's method. The results show that fixation for 2 hr in 30% acetic alcohol removes part of the histone. Hydrolysis at 60° for 10 min in N HCl completes the removal of this nuclear component. At the same time phosphorus corresponding with 27% of the original nucleic acid present is lost from the nucleus. Moreover, phosphorus containing substances can be detected analytically in the hydrolysis fluid, which also gives a deep violet colour when neutralized and treated with Feulgen's reagent and a blue colour when heated with Dische's reagent. These results accord with our interpretation of Feulgen's reaction. Their implications are discussed.

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Some Metabolic Products of *Trichothecium roseum* Link By G G FREEMAN and R I MORRISON (Imperial Chemical Industries Limited, Explosives Division, Research Department, Stevenston, Ayrshire)

The isolation and properties of trichothecin, an antifungal substance from the culture filtrate of *T. roseum*, have been described (Freeman & Morrison, 1948). Trichothecin, which inhibits the growth of many fungi but has no antibacterial properties, is a colourless crystalline unsaturated ketone, m p 118°, $[\alpha]_D^{25} + 44^\circ$. It has the probable molecular formula $C_{15}H_{20}O_4$.

In the course of fractionation of *T. roseum* culture filtrates three other crystalline metabolic products were isolated. These do not appear to have been described previously and will be referred to as *rosein I*, *II* and *III*, respectively. The three sub-

stances were present in the chloroform extract of the culture filtrate, *rosein I* and *II* have also been isolated from the dried mycelium in larger amounts.

Chloroform extracts of the mycelium consisted mainly of a mixture of lipids from which *rosein I* crystallized on evaporation of the solvent or on addition of petroleum ether, b p 80-80°, to the oily residue. It was purified chromatographically and obtained as colourless compact crystals, m p 210°. Yield, 3.2% of dry mycelium. *Rosein I* was readily soluble in chloroform and acetone and slightly soluble in ether, benzene and ethanol $[\alpha]_D^{25} - 113^\circ$,

$[\alpha]_{5461}^{25} - 144^\circ$ (c, 1 in chloroform) The analytical data were in agreement with the molecular formula $C_{19}H_{28}O_3$. The substance was a neutral ketone.

Rosein II was also obtained in highest yield from the chloroform extract of the mycelium. It was separated from rosein I by means of its greater solubility in ether and in boiling 50% (v/v) aqueous ethanol. It was also obtained by extraction of the oily residue with 75% (v/v) ethanol. It was purified by recrystallization from 50% (v/v) ethanol and from petroleum ether, b.p. $60-80^\circ$, from which it crystallized in colourless fibrous needles, m.p. 186° , yield 1.6% of mycelium. Rosein II was neutral and free from ketonic properties. $[\alpha]_D^{25} + 6^\circ$ (c, 2 in chloroform or ethanol), $[\alpha]_{5461}^{25} + 7^\circ$ (c, 2 in chloroform). Its analytical data agreed with the molecular formula $C_{19}H_{28}O_3$.

Rosein III was obtained from the chloroform extract of the culture filtrate in a yield of 100 mg/l and separated from trichothecin by chromatography on activated alumina. It crystallized from toluene as colourless compact crystals, m.p. 221° . The sub-

stance was slightly soluble in petroleum ether, b.p. $60-80^\circ$, and ether, but readily soluble in chloroform and boiling 50% (v/v) aqueous ethanol. $[\alpha]_D^{25} - 124^\circ$ (c, 1 in chloroform). Its analysis agreed with the molecular formula $C_{20}H_{28}O_4$. Rosein III was neutral and contained a ketonic group.

Roseins I and III had no antifungal or antibacterial activity. Rosein II was active against *B. subtilis* but had no antifungal activity.

The crude oil extracted from the mycelium was decolorized by passage through a column of charcoal. The oil was liquid at 20° and crystallized partially below 15° . It amounted to 20% of the mycelium. Its saponification value was 194. The corresponding fatty acids were separated into solid and liquid fractions by the lead salt method (Twitchell, 1921) and were found to consist of about 40% of solid and 60% of liquid acids. The latter consisted mainly of oleic acid, but the presence of linoleic acid was established by isolation of its petroleum ether insoluble tetrabromide, m.p. 115° .

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Two Crystalline Mycelial Constituents of *Trichothecium roseum* Link. By S. E. MICHAEL (Department of Biochemistry, London School of Hygiene and Tropical Medicine, London, W.C. 1)

A strain of the mould *Trichothecium roseum* Link, freshly isolated from an apple by Mr G. Smith, was found to grow somewhat slowly on Czapek-Dox or Raulin-Thom medium, but to grow well on Czapek-Dox medium with added cornsteep liquor.

The culture is harvested after 3 weeks' incubation at 24° on the latter medium. The yellow culture fluid contains a bitter principle which can be extracted with ether. Continuous extraction of the dried and powdered mycelium with light petroleum affords a mixture of two colourless, crystalline products (2-3% of the weight of mycelium). The two products are separated by treatment of the mixture with cold ethanol which leaves *product I*, m.p. 210° (approx. two thirds of the mixture), undissolved, while *product II*, m.p. 186° , crystallizes from the alcoholic liquors on addition of water.

On Raulin-Thom medium, only *product I* is formed. A preliminary examination of these products has shown the following facts.

Product I. Colourless pyramids (from alcohol), m.p. 210° . Analysis: C, 74.99, 75.01, 75.37; H, 8.70, 9.03, 9.00. No methoxy or active H present. Mol. weight (Rast) 316. Its empirical formula has not been established with certainty, but the formula $C_{19}H_{28}O_3$, appears possible, amongst others. The substance is soluble in most organic solvents, incl. hot petroleum ether. It is optically active.

$[\alpha]_{5461}^{20} = -140^\circ$ in chloroform (c, 1). It has no acidic properties. The presence of carbonyl is indicated by the formation of a yellow 2,4-dinitrophenylhydrazone, m.p. 237° . Heating with alkali in ethanol converts *product I* into an acidic substance, m.p. 143° , which has the same analysis, but is more soluble in ethanol than *product I*, and which has the rotation $[\alpha]_{5461}^{20} = +40^\circ$ in ethanol (c, 1). Oxidation of *product I* with $KMnO_4$ in acetone gives a substance, m.p. 242° , which, according to analysis and titration, appears to be a monobasic acid, $C_{16}H_{24}O_4$.

Product II. Colourless needles (from toluene), m.p. 186° . Analysis: C, 75.31, 75.37, 75.56; H, 9.48, 9.34, 9.21. No OMe or active H present. Mol. weight (Rast) 337. The substance is soluble in most organic solvents, more soluble than *product I* in cold ethanol and petroleum ether. It is optically active. $[\alpha]_{5461}^{20} = +7^\circ$ in ethanol (c, 1). Hot alkali slowly dissolves *product II* to a clear solution from which acid precipitates the unchanged *product*. $KMnO_4$ in acetone converts *product II* into an acid, m.p. 284° .

Comparison (by mixed m.p.) of the two crystalline products with the substances obtained by Freeman & Morrison (see above) showed that *product I* is identical with "Rosein I" and *product II* identical with "Rosein II".

Colorimetric Determination of Potassium by the Folin-Ciocalteu Phenol Reagent By M A M ABUL FADL

Determination of Serum Potassium by the Flame Photometer By W KLYNE (Department of Chemical Pathology, Postgraduate Medical School, London, W 12)

The potassium concentration of a solution may be determined by the flame photometer of Domingo, Klyne & Weedon (1948). The solution is sprayed into an air acetylene flame and the intensity of the 766 and 770 m μ K lines measured, using an Ilford 609 filter and a Cintel GS 18 photocell. A solution containing Na as well as K gives a higher galvanometer reading than a solution of the same K concentration containing no Na. Representative results for NaCl and KCl solutions are given in Table 1.

Table 1 Flame photometer readings for potassium

Solution	Concentration		Galv. reading*
	(mg K/100 ml.)	(mg Na/100 ml.)	
A	0	0	0.0
B	0	30	0.1
C	0	330	0.8
D	2.0	0	3.8
E	2.0	30	6.0
F	2.0	100	7.3
G	2.0	320	9.4
H	2.0	330	9.4
J	2.0	340	9.5
K	3.0	330	13.5
L	4.0	330	17.5

* Logarithmic scale

These higher galvanometer readings are presumably due to increased excitation of K by Na present in the flame (cf Brode (1943), regarding

the influence of extraneous elements on arc and spark spectra). Previous workers on the flame photometer (e.g. Barnes, Richardson, Berry & Hood, 1945; Hald, 1947) have not reported such interference, their flame temperatures were probably lower than that used in the present work.

For K determinations with our apparatus standard K solutions must be used containing Na in approximately the same concentration as the solutions to be tested. The dependence of the galvanometer readings on Na concentration decreases at higher Na concentrations as shown by solutions G, H and J in Table 1.

In serum K determinations variations in Na concentration from the normal (c. 330 mg/100 ml) are therefore minimized as follows. Serum (1.0 ml) is diluted with a NaCl solution containing 330 mg Na/100 ml (9.0 ml). The resulting solution has a Na concentration of 320–335 mg/100 ml even for the most abnormal sera. Comparison is then made with standard solutions containing 1.5, 2.0, 2.5, etc. mg K/100 ml and, in each case, 330 mg Na/100 ml.

Results for normal sera by this method and by the cobaltinitrite method of Abul Fadl (1948) usually agree within ± 2 mg K/100 ml. K added to normal serum can be recovered with an error of ± 1 mg/100 ml.

I am indebted to Mr R. A. Brennan for much technical assistance.

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The Polymyxins. A Related Series of Antibiotics derived from *B. polymyxa* By GEORGE BROWNLEE and TUDOR S. G. JONES (The Wellcome Physiological and Chemical Research Laboratories, Beckenham, Kent)

The name 'Aerosporin' was applied by Ainsworth, Brown & Brownlee (1947) to a chemotherapeutic antibiotic, selectively active against Gram negative organisms. Its pharmacology was further studied by Brownlee & Bushby (1948), who emphasized its

bactericidal nature. Its clinical application to whooping cough was described by Swift (1948) in a preliminary report. The polypeptide nature of at least part of the molecule is inferred from the detection and isolation of D-leucine, L-threonine

L diaminobutyric acid and an unidentified, optically active fatty acid of formula $C_9H_{18}O_2$ (Catch, Jones & Wilkinson, 1948)

'Polymyxin' was discovered in America by Stansly, Shepherd & White (1947) as an antibiotic product of *B. polymyxa*, the antibacterial nature of crude culture filtrates of this organism had been the subject of an earlier communication by Benedict & Langlykke (1947). The component amino acids of this antibiotic were simultaneously identified by Bell, English, Shepherd, Winterbottom, Bone, Fellows, Howard & Rogers (1948) and Jones (1948a) as leucine, threonine, serine and $\alpha\gamma$ diaminobutyric acid, while the same fatty acid was also present.

Comparison of the two antibiotics* revealed an identical antibacterial spectrum, but several interesting pharmacological differences. Even the purest samples of 'Aerosporin' are toxic to the mammalian kidney (Brownlee & Bushby, 1948) and efforts directed to removing this property chemically have so far met with little success. Polymyxin also has the nephrotoxic property (Brownlee, Bushby & Short, 1948). After efforts to eliminate the

* By the exchange of samples through Dr Carey of the Lederle Laboratories Division of the American Cyanamid Co

nephrotoxic action, which included chemical purification, and the use of protecting agents (Brownlee & Short, 1948), strain selection proved most productive and quickly provided a number of antibiotics with different chemical and pharmacological properties. At least two are free from nephrotoxic properties.

The recognition of this related series, of which 'polymyxin' was one, raised in acute form the question of nomenclature. The reinvestigation of the taxonomic derivation of *B. aerosporus* and *B. polymyxa* (Porter, McCleskey & Levine, 1937) has shown the two to be identical and confirms the latter as the specific name. It is therefore appropriate that the collective name for the antibiotics shall be polymyxin and agreement in this sense has been brought about between the British and American workers involved. 'Aerosporin' has thus been renamed polymyxin A and the 'polymyxin' of Stansly *et al*, polymyxin D, while the newly investigated antibiotics which are free from the nephrotoxic property are polymyxin B and polymyxin E. There is also described polymyxin C.

The chemical basis on which the classification rests is given in the accompanying paper (Jones, 1948b).

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The Chemical Basis for the Classification of the Polymyxins By T. S. G. JONES (Wellcome Chemical Research Laboratories, Beckenham, Kent)

Polymyxin A ('Aerosporin') has been shown to contain D-leucine, L-threonine (Jones, 1948a, b), L- $\alpha\gamma$ diaminobutyric acid and an unidentified, optically active, fatty acid of formula $C_9H_{18}O_2$ (Catch, Jones & Wilkinson, 1948a, b). The same acids, with serine as an additional acid, are present in polymyxin D (Bell *et al* 1948, Jones, 1948c, Catch *et al* 1948a, b). That free serine is not present as such in the polymyxin D preparation studied, is shown by partition chromatograms. In these,

polymyxin D moves at a speed, relative to the solvent front, faster than polymyxin A. When applied to the paper in admixture the two antibiotics separate, giving two spots. Similarly when the products of other strains of *B. polymyxa* of diverse origin are examined it is possible to distinguish a number of groups by their relative speeds on the paper. Further, when the products of the acid hydrolysis of these materials are examined on the chromatogram, differences are noted in their

qualitative composition. These differences are summarized in Table 1, where the amino acids corresponding with selected strains are shown together with the classes to which they have been assigned. Except for polymyxin E, which has the same qualitative composition as polymyxin A, the different classes show differences in their amino acid make up. The basis of the classification, therefore, is that the relative solubilities in two solvents, reflected in the relative speed on the chromatogram, and the qualitative amino acid composition, should differ for the different classes. It is not known at present whether polymyxins assigned to one class differ quantitatively amongst themselves, and whether all the strains which produce these are actually identical organisms. It appears, however,

that CN 1984 and CN 2002, which have been shown to give products indistinguishable by chromatography both of the intact and the hydrolyzed materials, are distinct strains (Francis & King, 1948). Until very recently, when one strain apparently gave a product which, as the intact material, separated into spots corresponding with both polymyxin A and B, no evidence has emerged that any one strain gives more than one class of antibiotic. The apparent dual nature of this preparation needs confirmation with purer material. The common feature of all these antibiotics appears to be the presence, in the molecule, of threonine, $\alpha\gamma$ diaminobutyric acid, a neutral non polar amino acid and, as far as has been investigated, a saturated, optically active fatty acid.

Table 1. *The amino acid components of the polymyxins*

Polymyxin	Culture no *	Leucine	Phenylalanine	Threonine	Serine	$\alpha\gamma$ Diamino butyric acid
A	1984 2002 121	+	-	+	-	+
B	2219 1419	+	+	+	-	+
C	2135 2136 2185	-	+	+	-	+
D	B71†	+	-	+	+	+
E	2184 2164	+	-	+	-	+

* Wellcome Foundation Culture Numbers

† Lederle Laboratories Division of the American Cyanamid Co

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The Chemistry of Polymyxin A ('Aerosporin') Isolation of the Amino-acids D-Leucine, L-Threonine, L- $\alpha\gamma$ -Diaminobutyric Acid, and an Unknown Fatty Acid By J. R. CATCH, T. S. G. JONES and S. WILKINSON (Wellcome Chemical Research Laboratories, Beckenham, Kent)

The presence in polymyxin A ('Aerosporin') of D-leucine and L-threonine (Jones, 1948a, b), and the isolation of DL $\alpha\gamma$ diaminobutyric acid (Catch & Jones, 1948), has established the amino acid composition of the polypeptide portion of this antibiotic. The present work has confirmed the presence of D-leucine and L-threonine by actual isolation of these amino acids. The use of picric acid for the isolation of the basic amino acid has been found

to be unnecessary and L- $\alpha\gamma$ diaminobutyric acid and DL $\alpha\gamma$ diaminobutyric acid have been isolated as the mono hydrochlorides in yields in the proportion 3:1 by direct fractional crystallization from alcohol of the residue left after removal of the hydrochloric acid from the ether extracted hydrolysis mixture. Fractional precipitation by pyridine of the mother liquors from the crystallization of the di amino acid gave pure L-threonine, and, from

the filtrate by addition of β -naphthalene sulphonic acid, the salt of D-leucine was obtained. This enabled the isolation of pure D-leucine. All of the amino-acids were thus obtained as the optically and chromatographically pure substances. L- α -Diaminobutyric acid has also been isolated in the above manner from polymyxin B.

Extraction of intact polymyxin A, B or D in strongly acid solution by ether gave only traces of soluble material. After hydrolysis by hot acid, but not by alkali in the cold, ether extraction of the product yielded the same fatty acid for the three

polymyxins. This acid may be purified via the *p*-bromobenzylthiuronium salt, m.p. 160–161°, or by fractional distillation under diminished pressure. The acid gave a crystalline amide, m.p. 93°, and a liquid methyl ester, b.p. 35–36°/0.01 mm Hg. Optical activity is shown by both the acid, $[\alpha]_{461}^{25} = +8.6^\circ$ ($c = 2.22$, ether), and the ester, $[\alpha]_{461}^{25} = +9.0^\circ$ ($c = 1.55$, ether). Analytical data for the salt, the ester and the amide, which are presented, are consistent with the empirical formula $C_9H_{18}O_2$ for the acid.

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Nucleic Acids in the Development of the Chick Embryo: Changes in Ribonucleic Acid Phosphorus (RNAP) and Deoxyribonucleic Acid Phosphorus (DNAP) in Heart and Liver
By J. N. DAVIDSON and I. LESLIE (*Biochemistry Department, St Thomas's Hospital Medical School, London, S.E. 1*)

Evidence has accumulated in recent years to support the view that the nucleic acids are related to growth and to the synthesis of protein. Studies on embryonic development have shown that there is a higher concentration of both types of nucleic acid in embryonic than in adult tissues (Brachet, 1941; Davidson & Waymouth, 1944), and that the ribonucleic acid (RNA) in the whole chick embryo reaches its highest concentration when protein catabolism is most active (Novikoff & Potter, 1948).

In the present investigation the heart and liver of the chick embryo were selected on account of their contrasting functions. Sampling occurred at random intervals over 12 months from eggs of pure bred Leghorn stock incubated at 37–38°. The whole body, heart and liver of 9–20 day embryos were rapidly weighed and stored in absolute alcohol at 0° until they could be analyzed for RNAP and DNAP. The analytical method was based on that of Schmidt & Thannhauser (1945), and phosphorus was determined over the various ranges by three methods (Berenblum & Cham, 1938; Fiske & Subbarow, 1925; and Allen, 1940).

The curves for total RNAP and DNAP per organ at different stages of development follow closely the shape of the curves for the fresh weights of hearts and livers.

One feature clearly emerges when the changes in

the RNAP/DNAP ratio are compared. The ratio for the heart steadily falls from a value of 3.3 at 10 days to 2.0 at 20 days. The ratio is therefore highest when the rate of growth is most rapid, and falls off as this diminishes. In contrast, the ratio in the growing liver does not decrease uniformly, but rises to 4.3 at 12–13 days and after a slight fall returns to its original value of 4.0 at 20 days.

The steady decline in the RNAP/DNAP ratio for the heart is seen to be the results of a fall in the mean RNAP concentration of 50 μ g P/100 mg fresh weight at 9 days to 42.5 μ g P/100 mg at 20 days, and a simultaneous rise in the DNAP from 15–17 μ g P/100 mg to 21 μ g P/100 mg. Liver RNAP concentrations fall from 100 μ g P/100 at 9 days to 80 μ g P/100 mg at 20 days, and the DNAP from 25 μ g P/100 mg at 9 days to 20 μ g P/100 mg at 20 days. It may be that the falling concentrations of both RNAP and DNAP in the latter is produced by the increasing content of glycogen or other cellular constituents in the liver cells.

In neither of these organs is there a marked increase in RNAP, DNAP or in the ratio of these two nucleic acids at the 14th–15th days when protein catabolism of the embryo as a whole is taking precedence over the fat and carbohydrate catabolism (Needham, 1942).

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The Occurrence of Uracil in Liver Ribonucleic Acid By J N DAVIDSON and W RAYMOND
(The Biochemistry Departments of St Thomas's Hospital Medical School and Glasgow University)

Uracil is well known to be one of the pyrimidine bases of yeast ribonucleic acid and its presence in the pentose nucleic acids of the pancreas and of tobacco mosaic virus is suggested by the work of Jorpes (1924) and of Loring (1939) respectively. Barnes & Schoenheimer (1943), from the nucleic acids of mixed rat organs, isolated a crystalline material from a fraction which might have contained either thymine or uracil, and stated it to be thymine. Its N content was 24.4%. The nitrogen contents of thymine and uracil are 22.2 and 25.0% respectively.

Liver ribonucleic acid (Davidson & Waymouth, 1944) was hydrolyzed by the method of Pentl &

Schoenheimer (1944) and the pyrimidine bases separated by the method of Hunter & Hlynka (1937). Cytosine was obtained as picrate and uracil as the free base.

Found C, 42.70, H, 3.63, N, 24.6% $C_4H_4O_2N_2$ requires C, 42.86, H, 3.57, N, 25.0%.

The ribonucleic acid from one animal source, liver, therefore contains uracil as one of the constituent bases and since the animal body contains more pentose nucleic acid than deoxypentose nucleic acid, it is probable that the material isolated by Barnes & Schoenheimer was uracil rather than thymine. Their analytical figures confirm this view.

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Amino-acids in Nuclear Proteins By J N DAVIDSON and R A LAWRIE (Department of Biochemistry, The University, Glasgow)

The material employed was 'chromosun' prepared from calf thymus and rat liver by the method of Mirsky & Pollister (1946) and fowl erythrocyte nuclei. After removal of nucleic acid by the Sevag procedure the whole protein so obtained was exhaustively extracted with HCl to remove histone, leaving a protein fraction corresponding to the chromosomin of Stedman & Stedman (1943) or the Tr Pr of Mirsky & Pollister (1946). Whole protein, histone and chromosomin were examined for amino acid content by paper chromatography and tryptophan was determined in each fraction.

Histones from all three sources differed from the chromosomins in containing lysine and more serine, whereas the chromosomins (but not the histones) contained glycine. The histones contained aspartic

acid, glutamic acid, lysine, serine, alanine, tyrosine, arginine, valine, leucine, isoleucine, phenylalanine, proline and an unidentified constituent, but fowl erythrocyte histone differed from the other two histones in containing less leucine, isoleucine, valine and phenylalanine. All three chromosomins contained aspartic acid, glutamic acid, glycine, serine, alanine, tyrosine, arginine, valine, leucine (isoleucine), phenylalanine, proline and two unidentified constituents. Whole protein contained amino acids corresponding to the sum of those in the constituent histone and chromosomin. A trace of cystine was found in whole protein from rat liver nuclei. Tryptophan was absent from, or present only in traces in, the histones. Thymus chromosomin contained 0.76% and rat liver chromosomin 1.20% of tryptophan.

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The Effect of Vitamin B Deficiency on the Testes of Mice By L A ELSON and P C KOLLER
(*The Chester Beatty Research Institute, The Royal Cancer Hospital, London, S W 3*)

Mice deprived of vitamins of the B group show decreased activity of the liver succinioxidase owing to depletion of the cytochrome c component of this enzyme system (Elson, 1947). Examination of the testes of these mice (M R C strain) has shown that spermatogenesis and sperm differentiation are affected, the process of differentiation being arrested with formation of giant cells containing many nuclei. This stage is followed by gradual arrest of meiosis in the primary spermatocytes. Maintenance for about 30 days on the deficient diet results in a complete suppression of cell activity in some seminiferous tubules, which are often denuded of all but one layer of spermatogenic cells. Recovery was studied in animals kept on the deficient diet for 20–25 days and fed again on normal diet.

Meiosis commences again 8–10 days after restoration of normal diet, but the division in several primary spermatocytes shows various abnormalities, the most common being lagging or displacement of chromosome bivalents. Aneurin appears to be the component of the vitamin B complex chiefly concerned in these effects. Similar but less intense changes in the testes of mice in chronic aneurin deficiency have been reported by Dunn, Morris & Dubnik (1947).

These findings, together with the observations of Koller (1946) on the golden hamster, show that temporary diet deficiency can lead to effects apparently identical with the so-called spontaneous changes in the genetical structure of cells. They thus indicate a mechanism by which these naturally occurring variations could arise.

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Some Properties of the Crystalline Anti-Pernicious Anaemia Factor By E LESTER SMITH,
L F J PARKER and K H FANTES

Observations on the Liebermann-Burchard Reaction By A P KENNY (introduced by J C EATON)
(*Clinical Laboratories, Victoria Infirmary of Glasgow, Glasgow*)

The factors influencing the development of the colour in the Liebermann-Burchard reaction have been studied, using a Spekker absorptiometer and a set of eight Ilford spectral filters.

The findings of other workers that the absorption curve for cholesterol solutions tested by the above reactions shows two peaks at 600–690 and 430 $m\mu$ is confirmed.

The temperature at which the colour is developed considerably modifies these maxima. Curves have been plotted of absorption against time of development for cholesterol solutions treated with reagents and incubated at various temperatures. Between 0 and 20° the rates of increase of absorption at 690 and 430 $m\mu$ are similar. At temperatures above 20°, however, a critical value is reached, depending on the concentration of cholesterol, beyond which the component absorbing at 690 $m\mu$ diminishes, and that at 430 $m\mu$ increases. Since the loss at 690 $m\mu$ is not paralleled by a loss at 430 $m\mu$, there is evidence of the conversion of one coloured product to another form. The rate of development and conversion of the colour increase with rise of temperature. At 20° the critical value is reached in 22 min, at 30, 40 and 50° it is reached in 8, 3 and

1 min respectively. At 0° practically no colour develops over a period of 30 min, but subsequent incubation at a higher temperature results in the development of the colour along that particular temperature curve. 'Room temperature' development results in a curve which approximates to that obtained by incubating at 30° and is due to the initial heat of the reaction.

The deleterious effect of exposure to white or ultraviolet light has been shown to be due to action upon the coloured product itself. The proportions of acetic anhydride and sulphuric acid used also affect the final colour.

With carefully controlled conditions a strictly linear relationship exists between absorption at 430 $m\mu$ and concentration of cholesterol when the reaction is allowed to proceed in the dark for 30 min at 40°. Since development of absorption at 430 $m\mu$ can be more readily controlled it is suggested that in estimating cholesterol itself by this reaction, measurement should be made at this wavelength rather than at 690 $m\mu$, as has been usual.

The response of cholesterol esters under similar conditions is now being studied.

Observations on the Clearance of Potassium by the Kidneys By H ELLIS C WILSON

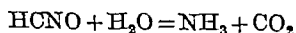
The clearance of potassium by the human kidney, appears in contrast to chloride, to vary inversely as the plasma potassium. After ingestion of potassium salts the plasma level tended to fall and the clearance rose sharply. It is suggested that the rate of potassium excretion is governed primarily by its concentration in the cells rather than the plasma level. Potassium taken by mouth may be taken up by the liver and/or other tissues, and

a stimulus possibly via the suprarenal may excite the kidney to excrete potassium until the concentration in the tissue cells is reduced. As potassium in contrast to sodium is primarily an intracellular electrolyte, the control of its excretion by the potassium concentration in the cells rather than the plasma level would appear to be more physiological.

Cyanase, an Enzyme Catalyzing the Hydrolysis of Cyanate By SHEILA B HOLTHAM and F SCHUTZ (Department of Pharmacology, University of Birmingham)

It was recently found that some pharmacological properties of cyanate, e.g. the diuretic action in rats (Schutz, 1946, Birch & Schutz, 1946), was lost on incubation of cyanate with organ extracts (Millington & Schutz, 1948). This was partly due to binding of cyanate by certain proteins (Holtham & Schutz, 1948), but the possibility remained of an enzymic destruction to have taken place as well. A further indication for the probable existence of a mechanism destroying cyanate in liver was obtained in recent experiments by Dirnhuber & Schutz (1947, 1948*a, b*), when small but significant amounts of cyanic acid were liberated and distilled from brain and other tissues, but no trace was recovered from either fresh liver tissue, or after it was incubated under physiological conditions.

An enzyme was found, capable of catalyzing hydrolysis of cyanic acid into ammonia and carbon dioxide, thus



The evolved CO_2 was measured manometrically. Aqueous centrifuged and filtered extracts of liver and kidney from freshly killed rats or guinea pigs were very active. Lyzed erythrocytes were less active. Serum, brain or muscle showed no activity at pH 6.3. The optimal pH (liver) was at pH 6.2-6.3.

At pH 5.5 the enzyme was inactive, and at 6.8 its activity was <40% of that shown at 6.3. The enzyme (liver and kidney) was thermolabile. Liver extract kept 15 min at 65° was inactive. CN^- was found to inhibit, KI, iodoacetate, H_2O_2 and NH_4^+ did not inhibit. Further properties of the enzyme are being investigated. We propose for this new enzyme the name of cyanase.

By means of the recently developed manometric method for the determination of cyanate (Dirnhuber & Schutz, 1948*c*), the described results have been confirmed. Moreover, it was possible to investigate the activity at pH >7. Liver extracts were still active at pH 7.4, though <30% of the activity shown at pH 6.3. There may be limitations to its activity *in vivo*. Tissue slices at pH 7.4 were still less active than extracts at the same pH. Since strong indications have recently been found for the formation of cyanate from urea in certain tissues (Dirnhuber & Schutz, 1947, 1948*a, b*, Holtham & Schutz, 1948), the physiological role of cyanase probably consists in catalyzing the hydrolysis of cyanate. It seems of interest that the substrate of cyanase is the simplest organic nitrogenous compound whose hydrolysis is catalyzed by a thermolabile enzyme. Pure sodium cyanate, made from urea according to Bader, Dupré & Schutz (1948), was used in all experiments.

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Toxicity of 2 3-Dimercaptopropanol (BAL) in Young Rats By J D P GRAHAM (introduced by J N DAVIDSON) (*Department of Materia Medica, University of Glasgow*)

Ten sets of young litter-mate white rats about 40 g in weight were arranged in six groups. They were given rat cube and water *ad lib*. To three groups arachis oil was administered daily 10 ml/kg, to the other three groups arachis oil in the same volume containing BAL 10 mg/kg. One control and one BAL-injected group were sacrificed at 14, 28 and 56 days after recording the weights of the rats daily. In a second similar experiment with three groups of young rats BAL 50 mg/kg was injected for 14 days. According to Dunn, Murphy & Rockland (1947), if one applies the formula $\log W = 1/t$ (Zucker, Hall, Young & Zucker, 1941, Zucker & Zucker, 1942) to the figures obtained by recording the growth of the rats over a period of weeks, a straight line graph indicates an adequate state of nourishment in the animals. This condition was found to apply both to the control and to the injected rats and the plots were parallel. There was no significant difference in the quantities of food eaten by the rats over the course of the experiment. Much larger doses of BAL than the 5 mg/kg which produces toxic effects in man (Modell, Gold & Catell, 1946) may thus be given to young rats

without affecting their appetite or rate of growth. The individual weights of thyroid, thymus, liver, kidneys, spleen, lungs, gonads, dried right femur, washed heart and washed gut similarly showed no statistically significant differences between the groups. X-ray of the skeleton showed no detectable difference in bone structure. Seifter & Ehrlich (1948) have shown that the thymus is a most sensitive indicator of general disturbance in growing rats. The thymus glands of rats injected with BAL 50 mg/kg for 14 days showed pyknonecrosis and were lighter by 4% than those of controls. The thyroid glands (190 $\mu\text{g/g}$ body wt) were heavier than in controls (125 $\mu\text{g/g}$) and showed histological evidence of a microfollicular hyperplasia of mild degree (% small follicles in controls 18%, in BAL treated rats 30%, 1000 follicles counted). The livers of injected rats showed some central necrosis and failure in glycogen storage.

It is concluded that up to 50 mg/kg of BAL is not very toxic to growing rats. There is only slight evidence of goitrogenic property in this compound and little evidence of thymus depletion or liver and kidney damage.

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DEMONSTRATION

Production of Streptokinase (Fibrinolysin) and Haemolysin by Haemolytic Streptococci
By J GREEN (*Gardner Institute of Medicine, Glasgow*)

The use of simplified media, coupled with strain selection, obviates many difficulties in the purification of bacterial products. A modification of Fildes's casein hydrolysate yeast extract medium has been used. Yields of streptokinase in this medium are comparable with those obtained with the usual digest and infusion media. Control of pH throughout growth is of cardinal importance (Christensen, 1945), the yield being negligible when the pH of the medium falls below 6.0. Within the pH range 8.0-6.5, streptokinase production parallels

bacterial growth under varying conditions of incubation.

Streptokinase can be recovered from filtrates by precipitation with alcohol or acetone, or 65% saturation with $(\text{NH}_4)_2\text{SO}_4$, by adsorption on alumina (Holmberg & Winblad, 1944) or by forming an ether-water interface.

The yield of haemolysin, on the other hand, is negligible in this simplified medium, and in dialyzed media (Krejci, Stock, Sanigar & Kraemer, 1942). In the heart-infusion and digest media used for

reference, streptolysin O (Todd, 1938) preponderated in the supernatants of infusion broth, while the haemolysin in digest broth was not oxygen sensitive. Preliminary work shows that a 'factor'

necessary for yields of the latter type of haemolysin can be removed by passing a calcium phosphate floc in the medium. The 'factor' can be recovered from the precipitate, and is non dialyzable.

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FORTHCOMING PAPERS

It is hoped to publish the following papers in the next issue of the *Biochemical Journal*

- Relations between hydrochloric acid secretion and electrical phenomena in frog gastric mucosa By E E CRANE, R E DAVIES and NORAH M LONGMUIR
- The effect of electric current on HCl secretion by isolated frog gastric mucosa By E E CRANE, R E DAVIES and NORAH M LONGMUIR
- Studies in the nitrogen metabolism of the apple fruit Changes in the nitrogen metabolism of the apple during the normal and ethylene induced climacteric rise in rate of respiration By A C HULME
- The algae 4 The lipochromes of the male and female gametes of some species of the Fucaceae By P W CARTER, L C CROSS, I M HEILBRON and E R H JONES
- Quantitative micro analysis of amino acid mixtures on paper partition chromatograms By A J P MARTIN and ROSE MITTELMANN
- The amino acid composition and titration curve of collagen By JOANE H BOWES and R H KENTEN
- The effect of alkalis on collagen By JOANE H BOWES and R H KENTEN
- The nutrition of the larva of *Aedes aegypti* Linnaeus
 3 Lipid requirements By L GOLBERG and B DE MEILLON
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Relations between Hydrochloric Acid Secretion and Electrical Phenomena in Frog Gastric Mucosa

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For over a hundred years the existence of a potential difference (p.d.) across the stomach wall has been recognized, and there has been sporadic speculation as to the relation, if any, between this p.d. and the secretory activity of the gastric mucosa. The p.d. was first recorded by Donné (1834) who, discussing these 'courants électriques' asked 'sont-ils capables de rapprocher, de séparer les molécules l'une de l'autre, et de donner naissance à des corps nouveaux, comme cela arrive dans le règne inorganique?' This question still remains unanswered in spite of a large amount of work by numerous investigators. The early experiments, using primitive techniques and uncontrolled conditions, have been reviewed by Biedermann (1895). Later work, some with more refined techniques, but still using the stomach *in situ*, have been discussed by Rehm (1944) and by Rehm & Hokin (1947). In the present investigations isolated frog gastric mucosa was chosen as the experimental material because it can secrete hydrochloric acid *in vitro*, under conditions capable of controlled variations, and because it is possible to obtain information about its metabolic activity (Davies, Longmuir & Crane, 1947). These investigations into a possible correlation between electric properties and metabolism were initiated in 1945, after it had become clear that the hydrogen ions of the acid could not be produced directly by the fermentation or oxidative degradation of any carbon compound (Davies, 1948, Davies *et al.* 1947).

This paper deals with the techniques and apparatus used, and presents the results of experiments which show that there is a general correlation between acid secretion and the p.d. across the mucosa, the resistance of the latter and its ability to produce electric power externally. This is taken to indicate that the elaboration of hydrochloric acid is an electrochemical phenomenon. A hypothesis of acid production is presented which is in accordance with the known biochemical and biophysical observations. Accounts of investigations into the effects of direct electric currents on acid production and the effects of metabolic activators and inhibitors will be given in subsequent papers.

A part of the present work has been communicated to the Biochemical Society (Crane, Davies & Longmuir, 1946).

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EXPERIMENTAL

Material. Frogs (*Rana temporaria temporaria* L.) were used in these experiments. They were captured locally, the

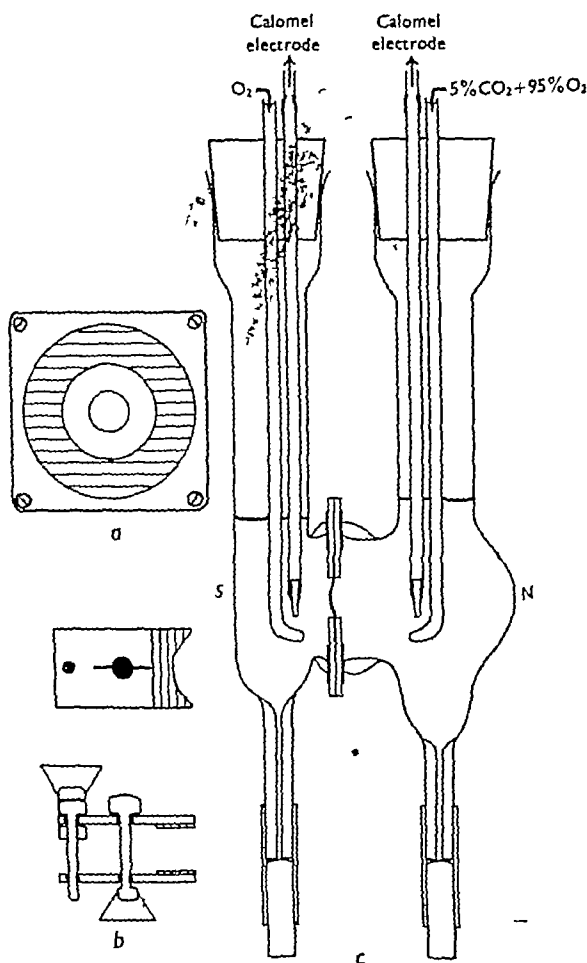


Fig. 1 a, Perspex holder for supporting mucosa, b, clamp for Type I and Type II vessels, c, Type I vessel for measuring p.d. across gastric mucosa and amounts of acid secreted. N = nutrient vessel, S = secretory vessel.

web tattooed for identification, and were housed in a concrete frogery at 15–17°. An excess of cockroaches or worms was provided for food.

Preparation and mounting of tissue The gastric mucosa was washed and isolated according to the method of Davies (1948) and the tube of mucosa then cut along the lesser curvature. Holders consisting of a pair of perspex sheets 1.5 mm thick and 4 or 5 cm square with a central hole were used for supporting the mucosa (see Fig. 1a). The holes, from 3 to 12 mm diameter, were surrounded on the inner sides by concentric grooves in the perspex which gripped the tissue. Rubber gaskets fixed on the outer sides prevented leakage at the joints with the glass vessels described below. The mucosa was laid across the hole between the two sheets and firmly clamped in position by tightening the four corner screws. In order to insulate it electrically the edges and screws of the holder were dipped in melted dental wax.

Each saturated KCl bridge consisted of two 15 cm lengths of 2 mm bore glass tubing connected by 30 cm of bicycle valve rubber tubing. The glass tubes were constricted at each end, and the lower end contained a tapered glass stopper 1 cm long ground into the tube. The narrow end of the stopper protruded a few millimetres beyond the tube (Fig. 1c). These bridges were designed because agar bridges, soaked strings and filter papers proved unsatisfactory. They were flexible and easily flushed, refilled and cleaned, and their leak was not more than 10^{-4} ml/hr with an excess pressure of 1 cm of saturated KCl solution. Their resistance was about 50,000 Ω .

The bridge from each vessel led to a test tube containing a reference electrode at 25.0°, and the p.d. between the two

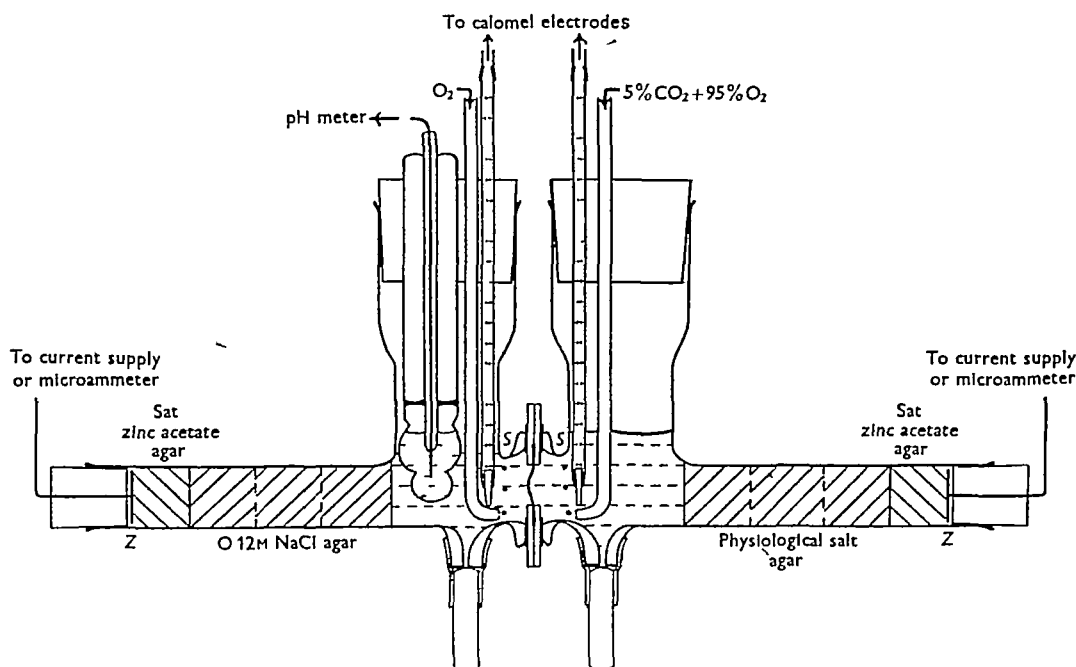


Fig. 2. Type II vessel for measuring pH of secretory solution, p.d. and resistance of mucosa, and for passing current through it. Oblique side tubes for locating bridges attached at SS. ZZ = zinc-zinc acetate electrodes.

Vessels The above unit was placed between the ground flanges of two glass vessels, which were then held together by a brass clamp with rubber-lined jaws (Fig. 1b). Several kinds of vessels were developed which were of two main types: Type I for measuring the p.d. across the mucosa and the amounts of acid secreted, and Type II for measuring additional quantities. Type I vessels are comparable with those of Delrue (1930) and of Gray, Adkison & Zelle (1940) and are shown in Fig. 1c. The nutrient vessel N contained about 36 ml. of nutrient solution (see p. 323) gassed with 5% $\text{CO}_2 + 95\%$ O_2 (v/v). The secretory vessel S contained about 14 ml. of 0.12M NaCl solution gassed with O_2 . There was an excess pressure of about 1 cm of solution on the nutrient side. The capillary draining tube at the foot of each vessel was closed by a glass rod attached by rubber tubing. Each bung supported by means of small rubber collars a funnel, a glass bubbler and one end of a saturated KCl bridge. The bubbler was a narrow glass tube delivering a fine stream of either O_2 or 5% $\text{CO}_2 + 95\%$ O_2 previously wetted and warmed to 25.0°.

Each saturated KCl bridge consisted of two 15 cm lengths of 2 mm bore glass tubing connected by 30 cm of bicycle valve rubber tubing. The glass tubes were constricted at each end, and the lower end contained a tapered glass stopper 1 cm long ground into the tube. The narrow end of the stopper protruded a few millimetres beyond the tube (Fig. 1c). These bridges were designed because agar bridges, soaked strings and filter papers proved unsatisfactory. They were flexible and easily flushed, refilled and cleaned, and their leak was not more than 10^{-4} ml/hr with an excess pressure of 1 cm of saturated KCl solution. Their resistance was about 50,000 Ω .

The bridge from each vessel led to a test tube containing a reference electrode at 25.0°, and the p.d. between the two electrodes was measured by a potentiometer described later (see also Fig. 3). In early experiments Ag/AgCl electrodes (Brown, 1934) in 0.1M KCl solution were used. Later, calomel electrodes were used in saturated KCl solution, the level of which was kept slightly above that in the vessels and storage test tube. The end A of each bridge was kept in this tube containing 0.12M NaCl solution at 25.0°, except during measurements. With this precaution diffusion potentials were satisfactorily eliminated. Checks were frequently made that the p.d. between the two reference electrodes was zero with both bridges connected by the same solution, and showed calomel electrodes to be completely reliable and more satisfactory than Ag/AgCl electrodes.

Type II vessels (Fig. 2) were designed for the measurement of the pH of the secretory solution, the p.d. and resistance of the mucosa, and for passing current through it by means of the non-polarizable zinc-zinc acetate electrodes ZZ. These were made by rubbing saturated Zn amalgam on to disks of pure cleaned Zn. They had a constant resistance for current densities up to about 50 ma/cm².

Before each experiment the horizontal tubes were filled with four layers of 5% agar, the first made with saturated zinc acetate solution and the other three with physiological salt solution (see p 323) or 0.12M NaCl solution. With this method no Zn was ever detected in the vessel solutions with 8 hydroxyquinoline or sodium diethyldithiocarbamate, and mucosae behaved normally. If all the saline agar was poured in together, so much zinc acetate dissolved in it that a toxic quantity subsequently reached the mucosa.

Bubblers were similar to those in Type I vessels. Bridges were similar, but for more accurate resistance measurements some of the Type II vessels were provided with side tubes inclined at 30° to the vertical (not shown in Fig. 2) which

mounted in the bath was 20–30 min. This period was found to be well within the safety limit for normal subsequent behaviour.

Saline medium. The solution used during the washing and preparation of the mucosa was similar to the physiological salt solution of Krebs & Henseleit (1932) diluted by 0.29 vol of water, except that it had a HCO_3^- content of 0.004M and, under working conditions, a pH of 7.4. The nutrient solution was that of Krebs & Henseleit diluted by 0.29 vol of water and containing 0.2% glucose. In aerobic experiments it was gassed with 5% CO_2 + 95% O_2 , in anaerobic experiments with 5% CO_2 + 95% N_2 . Analyses showed that our cylinders of this mixture contained 0.3–0.5% O_2 .

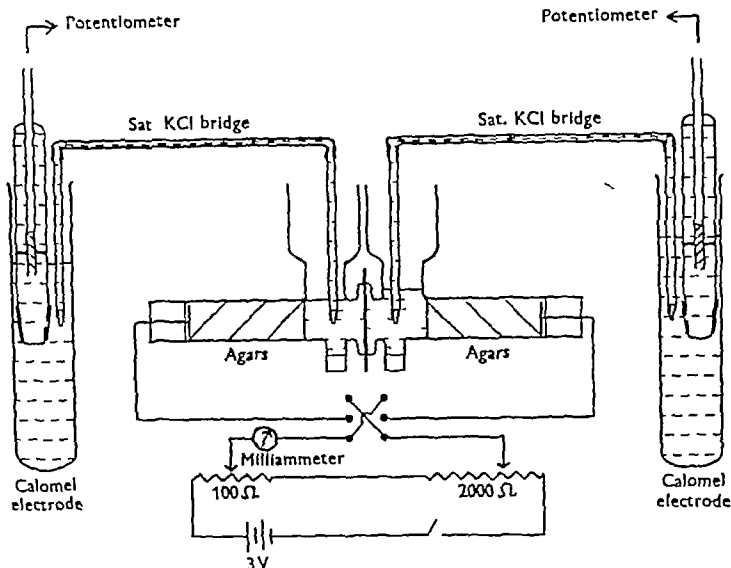


Fig. 3. Diagram of circuits for measuring p.d. and passing current through mucosa.

located the bridges accurately. This allowed precise determination of the correction due to the resistance of the solution. In experiments where a low correction was desirable, large Type II vessels were used, the horizontal tube having a diameter of 2.4 cm, nutrient and secretory volumes 25 ml. Where a low secretory volume was needed to give a higher acid concentration, vessels of 1.2 cm diameter were used, the nutrient volume was 7 ml, and secretory volume 4–6 ml. The glass electrode (Marconi TM 388 A) was mounted in the secretory vessel, on the far side of the bridge and bubbler from the mucosa. It was connected to a Marconi pH meter Type TF 5511C (see Fig. 2), and a saturated KCl bridge connected the secretory solution to a calomel reference electrode at 25.0°. The p.d. was measured as in Type I vessels.

Both types of vessel were immersed in a water bath at 25.0°, which also contained the reference electrodes, storage tube and gas manifolds. The temperature was kept uniform by water circulated through a warmed flask by air from a small motor blower, a second air stream stirred the water in the bath. In order to prevent the possibility of interference with electrical measurements electrical heating was avoided. The time elapsing between the putting of the frog and the commencement of the experiment with the vessels

Measurement of p.d. and resistance. When measuring the p.d. across the mucosa it is desirable that virtually no current pass through it. The p.d. recorded is then the e.m.f. of the mucosa, and the mucosa is unaffected by the measurement. Two potentiometers, both null instruments, were used in early experiments: a Cambridge Electrometer Valve Potentiometer 44236, and later a battery-operated Marconi pH meter and potentiometer Type TF 511C. The negative (secretory) side of the mucosa was connected to the grid of a valve and the positive (nutrient) side to the cathode circuit. The valve in each of these instruments has a very high grid cathode resistance, at least $10^8 \Omega$, so that the current through the mucosa was entirely negligible. The p.d. was determined to ± 1 mV, except in very early experiments when the accuracy was ± 2 mV. The resistance of the mucosa was determined by measuring the p.d. between the two saturated KCl bridges when a small current was passed through the membrane by means of the two zinc acetate electrodes. By measuring the p.d. between points near the mucosa the resistance correction was kept small, and by passing the current between electrodes covering the whole cross section of the tubes, uniform current flow was obtained. A potential divider provided currents from 0.01 to 10 ma (see Fig. 3), which were measured by multiple range meters.

with ranges from 0.1 ma upwards. Calibration showed them to be accurate to 1% (2% for lowest range). The value of the correction for the resistance of the solutions was calculated and also determined experimentally for each pair of vessels.

Estimation of acid secreted In early experiments the secretory solution was periodically removed and its acid content determined manometrically. In later experiments when a pH meter was available, the pH of the secretory solution was also measured *in situ* as described above. In the first method, a 6 ml. sample of the secretory solution consisting of 0.12M NaCl together with any secretion, also 0.12M, was placed in the main compartment of a Warburg cup, and 0.5 ml. of a solution containing 0.02M NaHCO_3 and 0.10M NaCl was placed in the side arm. After equilibration with 5% CO_2 + 95% O_2 at 25° the solutions were mixed and the output of CO_2 was observed. This determination, to about pH 6, was compared with those from control solutions of NaCl + HCl of total strength 0.12M, but of varying acidity. This procedure is necessary because it has been found that CO_2 is less soluble in acid than in neutral solutions of equivalent concentrations (Davies & Longmuir, 1947). The 'isotonic' NaHCO_3 was used to keep the blanks as low as possible.

The dry weight of the mucosa was obtained by removing the tissue quantitatively from the hole in the perspex holder, washing in distilled water and drying overnight at 110°. The Q_{HCl} was then calculated, and is given in $\mu\text{l}/\text{mg}$ dry wt./hr.

RESULTS

Electrical properties of non secreting gastric mucosa

Potential difference There was a p.d. across all of 150 frog gastric mucosae, the secretory side being in every case negative (in an external circuit) with respect to the nutrient side. Each experiment lasted between 6 and 12 hr. after the vessels containing the mucosa were mounted in the water bath, this instant being taken as zero time. During the first 0.5 hr. the p.d. between the secretory and nutrient sides usually increased in magnitude (Figs 6-8). We have found that after slight mechanical injury to either side of the mucosa the p.d. was temporarily reduced in magnitude by 2-15 mV (eight experiments). The initial rise is thus probably due to the decrease of injury potentials resulting from dissection and mounting, and we are not primarily concerned with it in these experiments. It occurred in seventy-two out of eighty-two experiments, the average initial p.d. of the secretory side was -20 mV and the average maximum value -32 mV. After this peak the p.d. dropped slightly and remained rather steady for many hours in an untreated mucosa unless acid secretion occurred.

In ninety-four experiments the steady p.d. varied between -7 and -50 mV, with a mean value of -30 mV and a standard deviation of 9 mV. There was no significant difference between males (69) and females (25), nor between fasting frogs and those actively digesting at the time of pithing. Nor was any correlation apparent with the weight of the

frog. There was, however, a difference between summer and winter frogs, in spite of the fact that the latter were kept warm and accepted food. From March to June 1946 the mean p.d. across thirty-seven mucosae was -35 mV and the standard deviation 7 mV, from September to February the mean p.d. across fifty-seven mucosae was -27 mV and the standard deviation 9 mV. Mucosae of frogs which had been in captivity over a month usually gave rather a low p.d., and were in general less active than those of freshly caught frogs. To overcome this difficulty frogs are now kept out of doors under natural conditions.

Resistance The resistance of 1 cm² of thirty-nine mucosae varied between 90 and 310 $\Omega\text{ cm}^2$, the mean value was 210 $\Omega\text{ cm}^2$ and the standard deviation 60 $\Omega\text{ cm}^2$. Taking the mean thickness as 0.25 mm, these results give for the mean specific resistance of frog gastric mucosa $8 \times 10^3\text{ }\Omega\text{ cm}$ and the standard deviation $3 \times 10^3\text{ }\Omega\text{ cm}$. It should be noted, however, that in all cases the area used was that of the hole in the perspex holder. Since the mucosa is essentially non-planar, these areas are minimum values, and represent between 0.5 and 1.0 times the true area. For this reason the unit of mucosa used in power calculations was not 1 cm² but 1 mg dry weight.

For both p.d. and resistance the standard deviation was about 30% of the mean value. No significant variation of the resistance for unit area or for 1 mg dry weight was observed with any of the variables mentioned in the last paragraph but one, nor with the area of mucosa used. Mucosae with a low specific resistance, however, never attained such high rates of acid secretion as some with high specific resistance (fifteen mucosae), and the magnitude of the specific resistance seemed to give rather a good indication of the 'state of health' of a mucosa at any particular time. The initial rise in p.d. was accompanied by a rise in resistance representing about 20% of its steady value (fifteen experiments). Injury to the mucosa reduced both p.d. and resistance. Death was accompanied by a rapid decrease of the p.d. to zero, and of the resistance to a small fraction of the live value. For mucosae killed by hydrochloric acid or hydrogen cyanide the dead resistance was 10-30% of the live resistance, for mucosae killed by heat (30 min. at 60°) not more than 5%. An interesting observation is the effect of iodoacetate. During 1-2 hr. after its addition to 0.0015M the p.d. was abolished and the resistance rose to about twice its normal value. Subsequently, the p.d. remained zero and the resistance dropped in 0.5 hr. to the dead value.

Electrical power output An important observation is the constancy of the resistance for currents below about 1 ma./cm². This was true for applied currents both enhancing and opposing the natural p.d.,

and also for currents maintained by the e.m.f. of the mucosa itself when no other source of power was included in the circuit. Results for one mucosa are shown in Fig. 4. As in all other similar experiments, the points obtained with an applied current, and those obtained when the mucosa itself provided the power, lie on the same straight line within experimental error, the mucosa obeyed Ohm's Law. Moreover, when the two sides were connected electrically (through the solution, agar, electrode and galvanometer resistances) a current was maintained without appreciable polarization. The greatest current observed when the mucosa was the only source of electrical power in the circuit was 0.07 ma. This occurred when the resistance in the circuit apart from the mucosa ($120\ \Omega$) was $460\ \Omega$. The electrical power output of the mucosa was then $(0.07)^2 \times 580\ \mu\text{W}$, or $2.8\ \mu\text{W}$. The effective area of the mucosa was $1.8\ \text{cm}^2$ allowing for bellying, and

Electrical properties of some other parts of the gastro intestinal tract

Various neighbouring parts of the digestive tract of the frog were examined. Across oesophageal, pyloric, duodenal and ileal mucosa (fourteen experiments) no p.d. was found comparable in magnitude with that across mucosa from the corpus of the stomach. The highest p.d. observed was that across the pyloric mucosa, which in eight experiments gave an average of $-5\ \text{mV}$. The other membranes had a mean p.d. of $-2\ \text{mV}$ (six experiments). In all cases, as in the corpus, the secretory side was negative with respect to the nutrient side. The resistance of oesophageal, pyloric, duodenal, ileal and rectal mucosa was determined in six experiments only; all values were within the range found for the corpus. This is also true of their specific resistance and resistance for 1 mg dry weight.

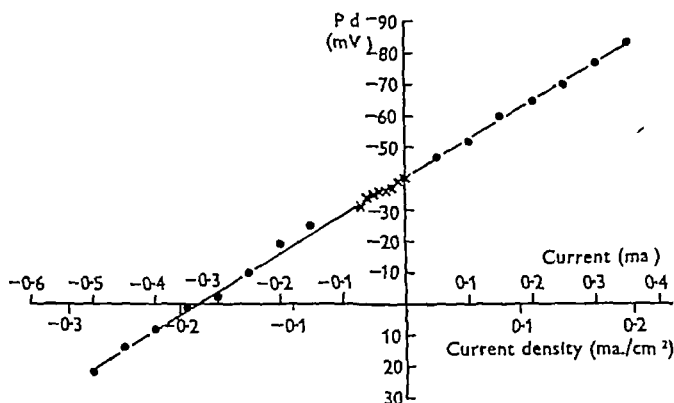


Fig. 4. Relation between p.d. across a frog gastric mucosa and current through it. \times — \times with mucosa as only source of power, \bullet — \bullet with external source of power.

its dry weight 10.8 mg. The greatest observed power output was thus $1.6\ \mu\text{W}/\text{cm}^2$ or $0.26\ \mu\text{W}/\text{mg}$ dry weight. The magnitude of the external resistance precluded the observation of a higher power output.

However, when sheets of mucosa were immersed in a beaker of nutrient saline solution at 25°C the p.d. across them, measured between two points close to them, was maintained without polarization at about one fifth of the open circuit value, so that the power developed was $11\ \mu\text{W}$. This suggests that if the resistance external to the mucosa could be reduced to zero, the total electrical power, $(\text{p.d. across mucosa})^2/\text{resistance of mucosa}$, would be developed. In the case considered above this maximum power would be $40^2/120\ \mu\text{W}$, i.e. $13\ \mu\text{W}$. This is also equal to $(\text{intercept on current axis})^2 \times \text{resistance of mucosa} = (0.33^2 \times 120)\ \mu\text{W}$. The maximum power is thus $7\ \mu\text{W}/\text{cm}^2$ or $1.2\ \mu\text{W}/\text{mg}$ dry weight.

Experiments with three rectal mucosae gave steady p.d. values of -8 , -12 and $-27\ \text{mV}$. The muscle layer dissected off from frog gastric mucosa was also investigated; the p.d. across it was zero in all of five experiments. The resistance of unit area was measured in three cases, giving a mean of $70\ \Omega/\text{cm}^2$. Since the thickness was about $0.5\ \text{mm}$ the specific resistance was $1400\ \Omega/\text{cm}$, considerably lower than that of the gastric mucosa.

Experiments in the absence of oxygen

Experiments on nine mucosae have shown that an adequate supply of oxygen is necessary to maintain both acid secretion and the p.d. across the membrane. Their mean p.d. with an oxygen pressure of 1 atm was $-27\ \text{mV}$, this was reduced to $-3\ \text{mV}$ when the oxygen was replaced by nitrogen (containing from 0.3 to 0.5% oxygen), and in four cases the p.d. fell to zero. The abolition of the p.d. was

reversible, and recovery occurred in *all* cases on supplying oxygen, even after several hours of anaerobiosis. The whole cycle could then be repeated using the same mucosa. When the experiment was begun under anaerobic conditions, there was no initial rise in p_d , which instead fell rapidly to a very low value as the oxygen in the tissue became depleted. Fig 5 gives a typical curve for two complete cycles.

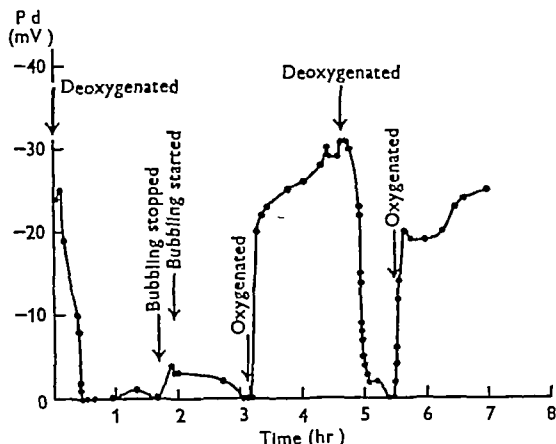


Fig 5 Effect of anaerobiosis on p_d across a frog gastric mucosa

The resistance of the tissue was increased in the absence of oxygen in both of two experiments by about 50%. It fell again to approximately its previous value on subsequent oxygenation. The secretion of acid by the mucosa was inhibited concomitantly with the decrease in p_d , and in no case was any hydrochloric acid produced anaerobically.

Secretion of acid by frog gastric mucosa

In dealing with the secretion of acid by frog gastric mucosa it was found that the seasonal variations in the behaviour of frogs both presented problems and gave a wide range of material from which to choose. In late spring and summer, about half the mucosae secreted acid spontaneously, and after the addition of histamine ($5 \times 10^{-5} M$) to the nutrient solution most others secreted acid. Histamine also often increased the rate of acid secretion of spontaneously secreting mucosae. The Q_{HCl} had values up to 8.0. In winter, fewer mucosae secreted acid and none did so spontaneously. The average rate was lower than in summer, and the maximum Q_{HCl} observed was 4.6. There was a period in spring, however, when summer conditions were not yet fully established, and almost no mucosa secreted spontaneously, but all did so after the addition of histamine, whenever this was added (usually between 1 and 7 hr experimental time, Figs 6-8). When spontaneous secretion occurred, it most often started at about 2 hr experimental time. In some winter and spring mucosae

secretion stopped on the removal of histamine, and secretion could subsequently be reinitiated by the addition of more histamine.

We wish to introduce the term 'secretive' for a mucosa capable of secreting acid, either spontaneously or during stimulation, whether this mucosa is actually secreting or not. The term 'non-secretive' is used for a resistant mucosa, i.e. one which cannot be made to secrete acid even by the addition of histamine.

No acid secretion was detected in oesophageal, pyloric, duodenal, ileal or rectal mucosa (eight experiments).

Relation between potential difference, resistance and acid secretion

The commencement of acid secretion, whether spontaneous or induced by histamine, was accompanied by a drop in the p_d across the gastric mucosa. This drop also accompanied a histamine-induced increase in the rate of secretion of a mucosa

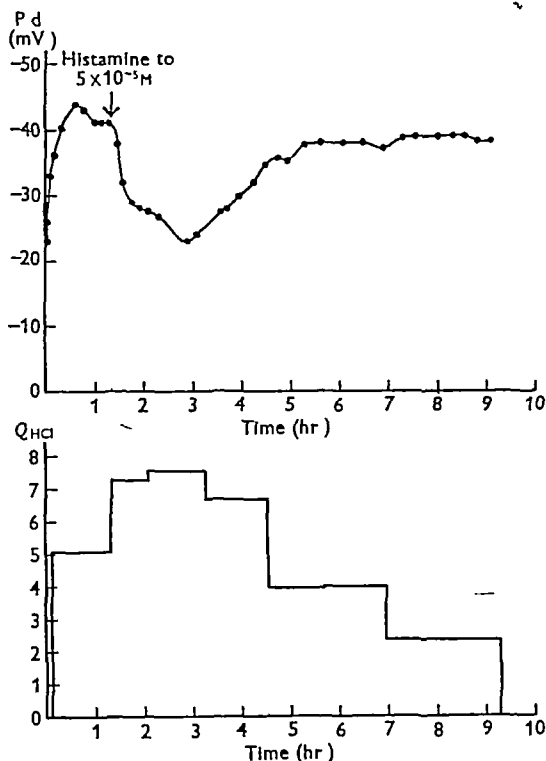


Fig 6 p_d and Q_{HCl} of a frog gastric mucosa. Spontaneous secretion and effect of histamine.

already secreting spontaneously. (In Fig 6 spontaneous secretion occurred during the initial rise in p_d , which masked the effect.) This decrease in magnitude of the p_d was usually higher for high rates of secretion than for low ones, being of the order of 10 mV for a Q_{HCl} increase of 2, or 17 mV for

a Q_{HCl} increase of 6. The effect was reversed when secretion stopped, either spontaneously (Fig 6) or on removal of histamine (Fig 8), or by the addition of thiocyanate (0.012M) to the nutrient solution, which inhibited acid secretion (Fig 7)

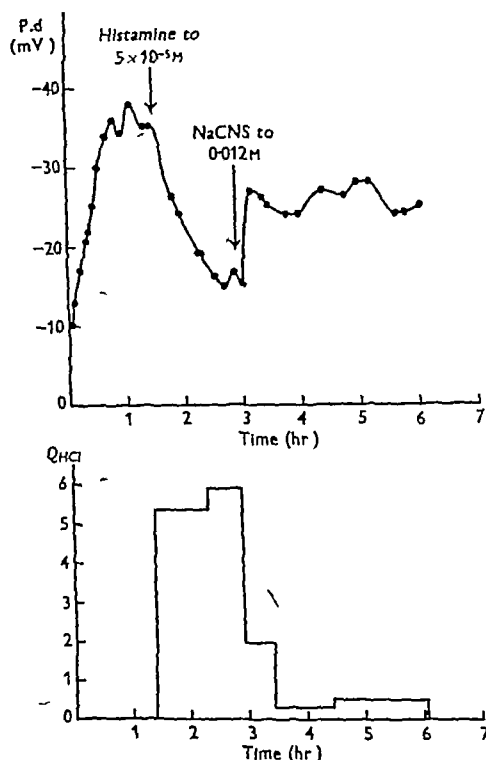


Fig 7 Effect of histamine and thiocyanate on p.d. and Q_{HCl} of a frog gastric mucosa

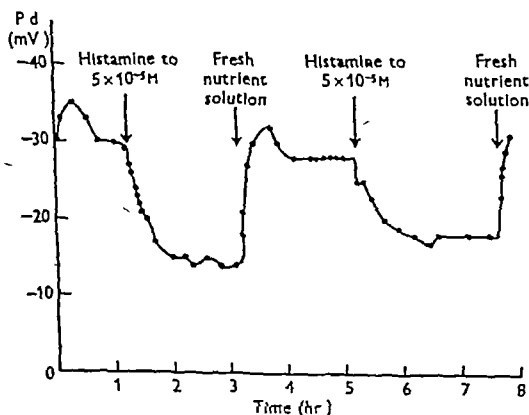


Fig 8 Effect of addition and removal of histamine on p.d. of a (winter) frog gastric mucosa

In summer, acid secretion sometimes commenced spontaneously before experimental time 0.5 hr, and in some cases it continued for periods up to 11 hr

Fig 6 gives results for a spontaneously secreting mucosa in April, which also responded to histamine and secreted for 9 hr, the rate falling off after the third hour. The maximum Q_{HCl} (over 1 hr) was 7.6 and the total amount of acid secreted 435 μ l. Fig 7 shows results for a secretive mucosa in March, which did not secrete acid until histamine was added at experimental time 1.5 hr. In this mucosa, acid secretion was almost completely inhibited after 1.5 hr by the addition of 0.012M thiocyanate which does not inhibit respiration (Davies & Turner, 1948), and the p.d. rose at the same time. Fig 8 refers to a mucosa in December which responded twice to histamine, the effect being reversed both times when the histamine was removed. In this experiment nutrient saline was used on both sides of the mucosa, and the acid was not estimated.

The variation of the resistance of the mucosa when acid secretion commenced (or was stimulated by histamine) was followed in six experiments, four of which were concerned with the actual onset of secretion. In all cases the resistance rose while the p.d. fell, and the fractional rise in resistance (x) increased both with the fractional fall in p.d. (y) and with the actual fall. In three cases x and y were both below 15%, in two cases both were between 30 and 40%, and in the remaining case $x = 20\%$ and $y = 11\%$. x and y were thus of the same order of magnitude in each case.

Diffusion potentials

The possibility was investigated that the change in p.d. across the mucosa at the onset of acid secretion is partly a diffusion potential due to acid in the tubules of the mucosa. Two types of experiment were carried out. (a) The 0.12M-sodium chloride on the secretory side of a non-secreting mucosa was replaced by 0.12M-hydrochloric acid, and after more than 10 min this was replaced by 0.12M-sodium chloride, the p.d. changes being watched carefully during the second change over. The 0.12M-hydrochloric acid left in the gastric crypts by this treatment is likely to take some minutes to diffuse completely into the new solution, and a p.d. level during this interval comparable with the p.d. after the onset of secretion would support the hypothesis stated above. No such temporary drop was observed. (b) A model was set up,

Hg | Hg_2Cl_2 | sat KCl | 0.12M NaCl | cellophane |
sintered glass disk | 0.12M NaCl | sat KCl | Hg_2Cl_2 | Hg,
to represent the system

Hg | Hg_2Cl_2 | sat KCl | nutrient solution |
muscularis mucosae | glandularis mucosae |
secretory solution | sat KCl | Hg_2Cl_2 | Hg

When the 'secretory' sodium chloride was replaced by 0.12M-hydrochloric acid the p.d. changed immediately from 0 to -20 mV and rose to -32 mV

during the next 5 min while the acid diffused through the sintered glass to the cellophan. After 1.5 hr the p d had fallen to -29 mV. When the hydrochloric acid was replaced by sodium chloride, the p d fell within 5 sec to -2 mV, and never changed sign, as would have been expected if the acid in the 'tubules' of the sintered glass caused asymmetrical diffusion potentials. In both these experiments care was taken to avoid momentary diffusion potentials at the bridges by rinsing the end in saturated potassium chloride solution before each p d measurement in a new solution.

It was important to know whether, at the pH values of the secretory solution obtained with secreting mucosae, diffusion potentials were produced which affected the measured p d. In six experiments hydrochloric acid solutions of pH 4, 3 and 2 were used consecutively on the secretory side of a non secreting mucosa in place of $0.12M$ sodium chloride. In no case did a solution of pH 4 or 3 produce any change in p d greater than 2 mV, whether or not it was made isotonic by the addition of sodium chloride. Hydrochloric acid ($0.01M$) produced no greater effect on mucosae when added within several hours of mounting, but in some cases increased the magnitude of the p d by up to 20 mV when added after 6 hr experimental time. These hydrochloric acid solutions had no effect on the p d of a secreting mucosa.

In our experiments the pH of the secretory solution never fell below 3, moreover, when the secretory solution of a secreting mucosa was replaced by fresh sodium chloride solution, there was no observable change in the measured p d. The secretory solution was always replaced before its pH fell below 3, and in view of all the above evidence it seems impossible to account for the p d changes during secretion on the basis of simple diffusion potentials.

DISCUSSION

✓ Sign of potential difference across gastric mucosa

Previous workers, including Rosenthal (1865), Bohlen (1894), Biedermann (1895), Mond (1927), Rehm (1943, 1944) and Teorell & Wersall (1945), found with the stomachs of a variety of animals that in an external circuit the secretory surface was at a negative potential with respect to the serosa, and that the p d diminished with the onset of acid secretion. Delrue (1935) claimed opposite signs for the p d and its change on secretion, it is probable that he was considering the direction of internal rather than external currents.

Injury potentials

The rapid increase in the magnitude of the p d across the mucosa after mounting is probably caused by the decrease of injury potentials which are in the

opposite direction to the natural p d. The accompanying increase in resistance is not great enough to account, by the reduction of local currents through the damaged edges as the tissue becomes stabilized, for more than a small fraction of the p d rise. Injury potentials of dog stomach have been discussed by Rehm (1944).

Relationship between potential difference, resistance and acid secretion

When acid secretion commenced, either spontaneously or after histamine, the magnitude of the p d fell by an amount greater for high than for low rates of acid secretion. The experiments on diffusion potentials support the view that not more than a small part, if any, of this fall can be due to a diffusion potential caused by the presence of hydrochloric acid either in the secretory solution or in the gastric tubules. It is clear from the data of Rehm (1944) and Rehm & Hokin (1947) that the pH in the secretory chamber attached to an actively secreting dog's stomach could fall from above 5 to 2 without any change in the p d across the stomach wall. Teorell & Wersall (1945) recorded only small changes in the p d across secreting isolated frog gastric mucosa when the pH of the secretory solution was changed from 4 to 2 by the addition of hydrochloric acid. They also noted that the secretory surface was 'insensitive towards the ionic composition of the solutions bathing it'. We also have found that wide variations in the ionic composition of the secretory solution had little effect on the p d across the mucosa. Similar results have been obtained by Rice & Ross (1947), who have reviewed the literature on this subject.

The rise in resistance which accompanies the p d drop at the onset of acid secretion, and seems to be quantitatively related to it, suggests that the electrical structure of gastric mucosa is changed when acid secretion commences, and that the p d fall is due to more fundamental changes than superimposed diffusion potentials. While many experimental procedures have been found which change both the resistance of live frog gastric mucosa and the p d across it, none was found which affected only one of these. Teorell & Wersall (1945) recorded a rise in both p d and resistance of isolated frog gastric mucosa between the time of mounting and the commencement of acid secretion about 1 hr later. They did not, however, differentiate between the injury effects due to mounting and changes accompanying the onset of secretion.

When acid secretion finished, either spontaneously or as a result of the addition of thiocyanate ($0.012M$) the magnitude of the p d rose again towards its previous value. Davies & Turner (1948) found that at this concentration thiocyanate had no effect on the rate of oxygen uptake of isolated frog gastric

mucosa, and that it neither prevented nor abolished the increased rate of oxygen uptake due to $5 \times 10^{-6} M$ histamine. In one experiment, when histamine was added to the nutrient solution of a secretive mucosa which already contained $0.01 M$ thiocyanate, the magnitude of the p.d. increased by 6 mV.

On the other hand, when both the cyanide sensitive respiration and acid secretion were abolished by $0.001 M$ cyanide, the p.d. fell to zero and the resistance fell to 10–30% of the original value. During anaerobiosis both respiration and secretion were also absent, but, whereas the p.d. fell rapidly to zero, the resistance increased by about 50%. On subsequent oxygenation the p.d. and resistance returned to their previous values even after several hours of anaerobiosis. Both Rehm & Hokin (1947) and Rice & Ross (1947) found that the p.d. across a dog's stomach wall was dependent upon an adequate oxygen supply to the tissue. No workers have recorded acid secretion in the absence of respiration or a p.d. across the mucosa.

Our results further show that the dead/live resistance ratio for isolated frog gastric mucosa may be considerably greater than for other active biological material for which information is available. Cole (1947) gives the value for the dead/live membrane resistance of cells as a fraction of 1%. Blinks (1930) found that the dead/live resistance for *Valonia ventricosa* cells was 1 or 2%. The highly active oxyntic cells in gastric mucosa may, however, have a different dead/live resistance ratio from the whole tissue.

Potential difference and acid secretion of other regions of the gastro intestinal tract

The p.d. across the oesophageal, pyloric, duodenal and ileal walls, and across the muscle layers dissected off the corpus of the stomach, was found to be small or zero and unaffected by histamine. It is of interest that of these regions only the acid secreting mucosa had a high p.d., and that the p.d. was affected by histamine only if it was secretive.

Experiments on rectal mucosa are being extended, and will be discussed in a later paper. It should be borne in mind in connexion with the appreciable p.d. of this tissue (mean of three experiments ~ 16 mV), that frog gastric mucosa, frog skin (which shows an even higher p.d.) and frog rectal mucosa are all tissues concerned actively with water transport. The last two membranes transport an approximately neutral solution in the same direction as their external positive p.d., whilst gastric mucosa secretes an acid solution in the opposite direction to its external positive p.d.

In frogs, pepsin is largely or entirely secreted by the oesophagus (see Friedman, 1937). In Friedman's (1934) experiments with frogs the brain was destroyed and 'one third of the way up from the pylorus the

stomach was divided to form a pyloric portion and a cardiac portion'. He found that when either the cardiac or pyloric part was stimulated with glass beads, acid was formed only in the pyloric region. No figures were published to support this claim. Using the technique described above, we have shown beyond any doubt that the corpus of the stomach produces acid, and have also failed to detect any acid secreted by the pyloric mucosa of a stomach whose corpus secreted acid. Friedman (1934) used *Rana esculenta*, and we have confirmed that in this species too acid is secreted by the corpus region of the stomach.

Electric power produced by isolated frog gastric mucosa

Donné (1834) first raised the question as to whether the electric currents observed in the stomach could do useful chemical and mechanical work. Brücke (1859) suggested that the electromotive forces in gastric mucosa may enable it to send an acid fluid towards the secretory surface and a basic fluid in the opposite direction. In 1872 Engelmann published experiments which are of interest in connexion with these suggestions. He studied the secretion of the glands in frog skin by drying the skin and observing microscopically when it became wet again. Although he did no quantitative work he showed that the glands secreted only when an e.m.f. could be detected, and that various agents which abolished the e.m.f. also abolished secretion. He suggested that the glands in the skin produced their secretion by electroendosmosis (Engelmann, 1872). Since then there has been much speculation, but until recently very little experimental evidence has been published, and this is more suggestive than conclusive. Some of the work has been reviewed by Keller & Pisha (1947).

Recently evidence has accumulated which supports the hypothesis that electrical energy is utilized in the production of acid by the stomach. It has been found that the rate of acid secretion of gastric mucosa can be increased by passing through it an electric current which enhances the natural p.d., and decreased by a current in the opposite direction (Rehm, 1945 (dogs), Crane *et al.* 1946, Crane & Davies, 1947a (frogs)). It has also been found that gastric mucosa can maintain an electrical power output without appreciable polarization (Rehm, 1943, Crane *et al.* 1946). The greatest observed power output of the frog gastric mucosa considered above (p. 325 and Fig. 4) was $0.25 \mu W / mg$ dry weight, and the power output expected with zero external resistance was $1.2 \mu W / mg$ dry weight. Davies & Longmuir (1947) found the average Q_{O_2} of fifty non-secreting isolated frog gastric mucosa to be 2.1. If we take as a first approximation the usual value of $0.005 \text{ cal} / \mu l$ oxygen for the oxidation of glucose (Brody, 1945), the average rate at which energy is

liberated in non secreting frog gastric mucosa by glucose oxidation is $0.0050 \times 2.1 \text{ cal/hr/mg dry weight}$, or $12 \mu\text{W/mg dry weight}$ ($1 \text{ cal/hr} = 1.16 \times 10^3 \mu\text{W}$). Using this value for the mucosa discussed above, the greatest electrical power output observed represents $0.25/12$, i.e. 2% of the metabolic power, which with zero external resistance would be increased to $1/2/12$, i.e. 10%.

mately 10%) in maintaining an electric current. This refers to a non secreting mucosa and, since the p_d is lower and the resistance higher during acid secretion, less power is then available to drive a current round an external circuit. This may be because electric power is being used internally to produce acid. The maximum electric power output of the mucosa considered earlier (p. 325), which was

Table 1 *Production of maintained electric power by various living tissues*

Author	Material	Temperature (°C)	Approximate p_d across membrane (mV)	Greatest observed current density in active membrane (ma/cm ²)	Greatest observed electrical power output of active membrane ($\mu\text{W/cm}^2$)	Greatest observed electrical power output of active membrane ($\mu\text{W/mg dry wt}$)	Electrical power / Metabolic power (Author's figure) (%)
Blanks (1933)	Halcyon cell	25-30	70	0.01 ma/cell	$0.7 \mu\text{W/cell}$	—	—
Francis (1933)	Isolated frog skin	20	—	0.015	2.5	0.5	5-10
Stapp (1941)	Isolated frog skin	25	40	0.02	0.8	0.15	1-2.5
Rehm (1943)	Dog stomach	39	60	0.15	8.9	0.34	2-4.5
Crane <i>et al</i> (this paper)	Isolated frog gastric mucosa	25	40	0.04	1.6	0.26	2

At least five workers besides ourselves have determined the electrical power output maintained by living tissue, and some of the results are collected in Table 1. (The specialized tissue of electric organs of some fishes is excluded from the present discussion, the current varies rapidly and widely during a train of discharges.) All the results in Table 1 refer to experiments in which the two sides of the membrane were connected electrically through a circuit of fairly low resistance, and the current densities cited were maintained without appreciable polarization. Throughout, the power produced was limited by the external resistance, which differed in each case, and a higher power would be expected with zero external resistance. The last column gives an approximate value of the ratio: greatest observed electrical power output/estimated rate at which metabolic energy is liberated (the efficiency of the process). Whilst some of the figures in this table are based on incomplete data, it is of interest that the efficiencies are of the same order of magnitude. Lund & Stapp (1947) have recently published a series of results for frog skin, which give efficiencies (electrical power/metabolic power) of 1-2%.

From a comparison of the structure, the p_d and resistance of nearby regions of the gastro-intestinal tract, and from the relations previously described between the electrical properties of gastric mucosa and acid secretion, it seems likely that most, if not all, of the p_d across this tissue is due to the oxyntic cells, but that their resistance is only a small part of the whole.

It is remarkable that a gastric mucosa can expend such a large fraction of its metabolic energy (approx-

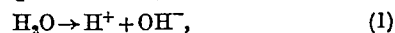
imately 10%) in maintaining an electric current. This refers to a non secreting mucosa and, since the p_d is lower and the resistance higher during acid secretion, less power is then available to drive a current round an external circuit. This may be because electric power is being used internally to produce acid. The maximum electric power output of the mucosa considered earlier (p. 325), which was

A SUGGESTED ELECTRON CYCLE MECHANISM OF HYDROCHLORIC ACID PRODUCTION

Golgi (1893) found that the intracellular canaliculi form a network just below the surface of the oxyntic cells (shown schematically in Fig. 9b). Bradford & Davies (1948), using indicators, demonstrated that these canaliculi contained acid during acid secretion, thus showing that the acid is formed in the region between the canaliculi and the external cell wall (pericanalicular zone).

Chemical processes involved

The fundamental overall reaction concerned in hydrochloric acid production (Davies, 1948) is



and evidence has been presented in this paper and previously (Rehm, 1943, 1945, Crane *et al.* 1946, Davies *et al.* 1947, Crane & Davies, 1947a) which supports the theory that some of the energy available from metabolism within the oxyntic cells is utilized in an electrochemical process which results in the net separation of the electrically charged H^+ and OH^- ions.

We suggest that the cyclic mechanism described in the following paragraph, and shown in Fig 10, is

According to our hypothesis two coupled systems of enzymes are involved (a) to transport H atoms

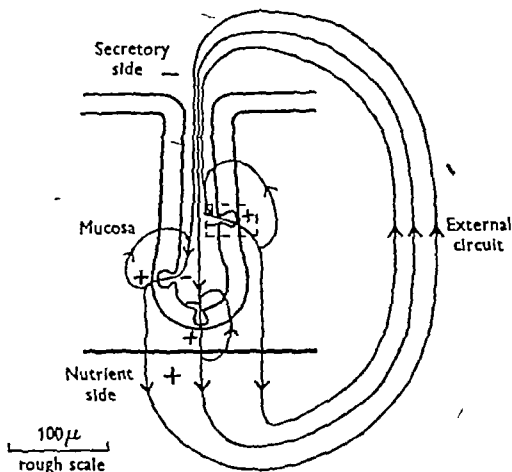


Fig 9a.

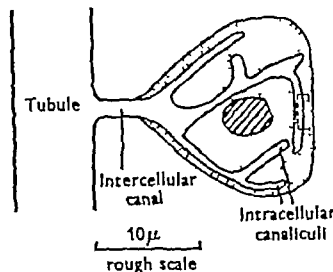


Fig 9b

Fig 9 a, Diagram of gastric tubule showing lines of current flow across and in the mucosa due to its own e.m.f. Three oxyntic cells shown b, Diagram of single oxyntic cell enlarged from a showing network of canaliculi. Sample cross section of pericanalicular zone enclosed in rectangle

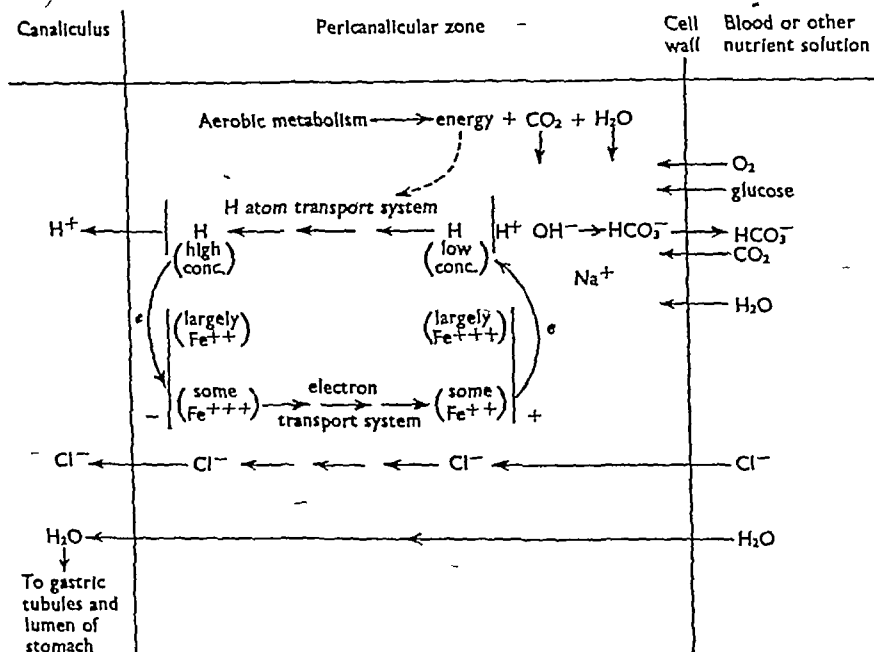
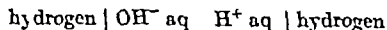


Fig 10 Suggested electron cycle mechanism of HCl production in the pericanalicular zone of oxyntic cells (enlarged from Fig 9b). This schematic diagram is not drawn to scale, the H atom and electron transport systems probably occupy only a small fraction of the space between the canaliculus and cell walls

operative. The overall reaction is that which occurs when the well known cell



(see MacDougall, 1939) is reversed by a source of power (Davies *et al* 1947)

(electrons + protons) across the pericanalicular zone, and (b) to return the electrons to the starting point near the cell wall. Energy derived from aerobic metabolism drives a system composed of a series of dehydrogenating enzymes (a) whose prosthetic groups (such as coenzymes I and II) send H atoms

through the pericanalicular zone, thus maintaining a very low H atom concentration at the side nearest the cell wall and a very high H atom concentration at the canalicular wall. The electrons are returned by a coupled cytochrome or cytochrome like system (b) (e.g. $\text{Fe}^{++} \rightarrow \text{Fe}^{+++}$) oriented across the zone. A unit cycle can now be described. A H^+ ion formed from water near the cell wall oxidizes a Fe^{++} to a Fe^{+++} ion, and by accepting the electron becomes a H atom. The atom is transported to the region of high H concentration at the canalicular wall, this endergonic process being coupled with energy giving reactions. The H atom then reduces a Fe^{+++} to a Fe^{++} ion, the resultant H^+ ion passes into the canaliculus together with the Cl^- ion released by reduction of the Fe^{+++} ion. The electron from the H atom is returned to the region of low H concentration near the cell wall by the potential of the $\text{Fe}^{++} \cdot \text{Fe}^{+++}$ system, and passes round the cycle again. Valency requirements are satisfied by the passage of a Cl^- ion across the system in the opposite direction to the electron (cf. Lundegårdh, 1945). The overall result for each electron cycle is thus reaction (1) with the transport of one Cl^- ion.

Previous work has shown that the residual OH^- ions from the water react (via the cytoplasmic buffers) and are converted by carbon dioxide from internal and external sources into HCO_3^- ions (Davies, 1948, Davies & Longmuir, 1948), this process being catalyzed by carbonic anhydrase (Davies & Roughton, 1948). It is clear, therefore, that this cyclic mechanism involves no net transfer of charge through the acid-secreting oxyntic cells or across the mucosa at the cell wall Cl^- ions exchange with HCO_3^- ions, and at the canalicular wall H^+ and Cl^- ions are secreted (cf. Davenport, 1946).

Effect of histamine The action of histamine could be explained by the assumption that it takes part as a link in the H atom transport system of the electron cycle by the tautomerism or resonance of its iminazole ring. A similar role for histidine has been suggested as part of the mechanism of cytochrome c activity (Theorell, 1947). The work of Hallenbeck, Code & Gregory (1947) supports the view that agents stimulating hydrochloric acid secretion act finally through histamine (cf. Babkin, 1944).

Crane & Davies (1947b) have calculated that in order to form 1 mol. of hydrochloric acid under the conditions obtaining in frog oxyntic cells, the system must be supplied with about 1.0×10^4 cal. of free energy (a few hundred calories more are required in mammalian oxyntic cells). Lipmann (1941) and Meyerhof (1944) have calculated the free energy released by the hydrolysis of high energy phosphate bonds. For ATP they found c. 10,000 and 12,000 cal./mol., respectively. It is interesting that the splitting of one such bond might thus be able to energize one unit electron cycle and thus produce one H^+ ion.

Electrical processes involved

Potential differences in oxyntic cells The postulated electric circuits within the mucosa are shown schematically in Fig. 11a and b, which represent a sample cross section of the mucosa containing a single secretory unit in the pericanalicular zone of an oxyntic cell. E is the e.m.f. which maintains the p.d. measured in our experiments.

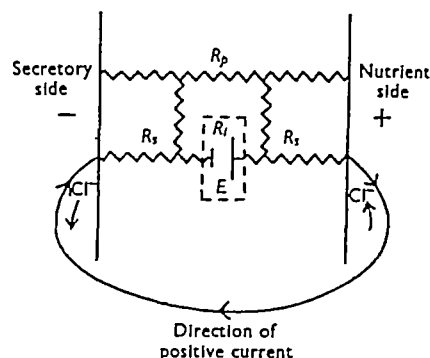


Fig. 11a

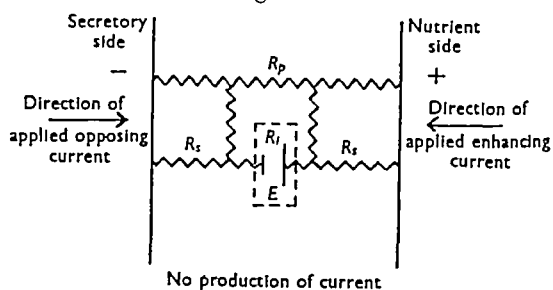


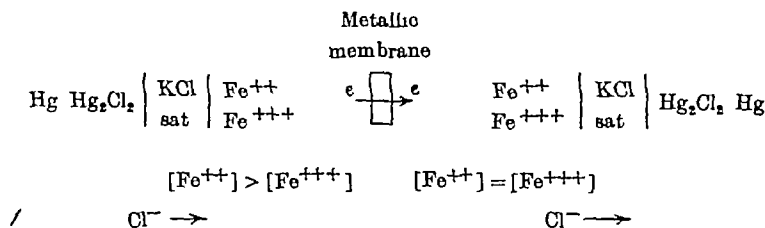
Fig. 11b

Fig. 11 Suggested electric circuit diagram within sample cross section of gastric mucosa. Enclosed portion represents the pericanalicular zone of the single oxyntic cell considered. a, Non secreting mucosa (showing external current). b, Acid secreting mucosa. E = e.m.f. of pericanalicular zone. R_i = internal resistance of pericanalicular zone. R_s = mucosal resistance in series with R_i . R_p = resistance of rest of sample cross section of mucosa.

Since the pH difference across the pericanalicular zone of a frog oxyntic cell secreting 0.12N hydrochloric acid is c. 6.3 (calculated from Davies & Roughton, 1948), the p.d. between the two sides of this zone, measured with respect to a reference electrode, must be about 370 mV. This p.d. is measurable by electrodes sensitive to H^+ ions only (e.g. glass electrodes). Since the p.d. occurs across a distance not greater than 1μ (Fig. 9b), the electric field is at least 3700 V/cm between the canaliculus (positive) and the cell wall (negative). If the hypothesis presented above be true, this p.d. due to the pH difference during acid secretion must be more than compensated by that due to the difference

in H atom concentration. Assuming that the H atoms are in equilibrium with hydrogen molecules, then electrodes sensitive to both H_2 and H^+ ions (i.e. hydrogen electrodes), if used together with a reference electrode, would detect a p.d. in the opposite direction, from cell wall (positive) to canaliculus (negative). On this hypothesis the ratio of the equivalent hydrogen pressures must be more than $10^3 \times 10^3 = 10^{12.6}$ (see MacDougall, 1939). Such ratios certainly occur within living cells (Hewitt, 1936, Ball, 1942).

There has been controversy (see Korr, 1939) as to whether redox potentials can be the direct source of bioelectric potentials measurable with non-metallic, i.e. calomel, electrodes. This has been resolved by Korr (1939), who pointed out that the redox potential of the following system can be observed by calomel electrodes

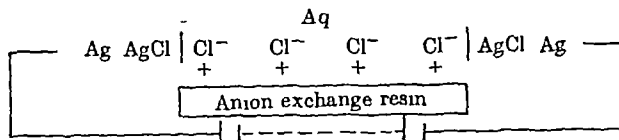


and that if a system able to transfer electrons existed in the cell (as we postulate for the oxyntic cell) then redox potentials could be manifested as bioelectric potentials. Stiehler & Flewener (1938), using indicators, measured the difference in redox potential in the choroid plexus and found it to be 230 mV with epithelium positive to stroma. Similar results were obtained by Friedenwald & Stiehler (1938) for the ciliary body. It is of interest that, on the basis of these and other results, they suggested that these redox potentials can do secretory and electrical work.

Production of current by tissue. The production of electric power by living tissue is a major problem in itself. If the secretory units in the pericanalicular zone of the oxyntic cells function by the mechanism proposed above, they do not produce a current when actually secreting acid, but can do so when there is no net production of H^+ , OH^- (and hence HCO_3^-) ions. In this case the H transport system is modified and the H^+ ions, instead of passing into the canaliculus, return and react with OH^- ions to reform water. This part of the hypothesis is very similar to the 'osmotic diffusion pump' theory published by Franch & Mayer (1947).

The transport of Cl^- ions by the non acid secreting cell involves a net transfer of charge, the circuit being completed by conducting pathways inside and outside the mucosa. It can be observed as an electric current through a circuit external to the mucosa, in which the negative sign of the secretory side is determined by the morphological structure of the

tissue (Fig 9a). Experiments are in progress in an attempt to determine whether the current consists of Cl^- ions alone, as in the system



or whether cations move in the opposite direction. In this latter case the mucosa must be able to move cations such as H^+ , Na^+ , etc., from the secretory to the nutrient side.

Potential differences observed. In our present technique we measure the p.d. across the whole mucosa, with saturated calomel electrodes connected to the nutrient and secretory solutions by saturated potassium chloride bridges. It seems

likely that the average value of 30 mV observed across non secreting mucosa is less than the average p.d. across the pericanalicular zones theoretically observable with calomel electrodes, because of conducting pathways within the mucosa (see Fig 11a). For reasons already given, we consider it probable that the redox potential of the Fe^{++} - Fe^{+++} or similar system which transfers the electrons is the source of this p.d. across the pericanalicular zone. Each secretory unit contributes to the p.d. measured, and there are probably of the order of 10^{13} secretory units in each cm^2 of frog gastric mucosa (Crane & Davies, 1947b). This figure is based on the known structure and activity of the tissue, and on an assumed turnover number of 10^3 H^+ ions/secretory unit/min (see McIlwain, 1946). As each secretory unit commences to secrete acid (Fig 11b), then according to the hypothesis there is no longer any net transfer of charge through it, i.e. it is unable to produce a current. Since fewer non secreting units are now available to maintain the p.d. across the mucosa, whilst the other conducting pathways remain, the p.d. observed externally should fall as more and more secretory units start to produce acid. The experimental results show that such a fall in p.d. occurs. As previously suggested, in the secreting unit the internal electron current, the magnitude of which is affected by the increased metabolic activity of the oxyntic cell, is effective in elaborating the acid secretion, but for reasons given above does not produce a p.d. across the mucosa.

Changes in electric resistance In non-secreting units the production of a Cl^- ion is believed to be concomitant with the transfer of an electron across the $\text{Fe}^{++}\text{-Fe}^{+++}$ system. Since during the remainder of the electron cycle there is no net production of H^+ ions, the process is likely to have a low energy requirement and be easily increased or decreased by externally applied electric p.d., i.e. the non acid-secreting unit will have a low electric resistance. As has been discussed above, the acid secreting unit produces hydrochloric acid but not current as a result of the cyclic movement of electrons. The same externally applied electric p.d. can now send very little current through the secreting unit, because the Cl^- ions forming the current must be forced across the $\text{Fe}^{++}\text{-Fe}^{+++}$ system independently of, and in addition to, the electron cycles. Thus the observation of an increase of electric resistance of the mucosa on secretion is in accordance with the hypothesis.

Effect of applied currents The increase in the rate of acid secretion by the action of applied direct electric currents enhancing the natural p.d. and the decrease by opposing currents (Crane *et al.* 1946, Davies *et al.* 1947, Crane & Davies, 1947a, Rehm, 1945) has been mentioned earlier. On the basis of our hypothesis an applied p.d. from cell wall (positive) to canaliculus (negative) could act in two ways, by assisting the return of electrons in the $\text{Fe}^{++}\text{-Fe}^{+++}$ or similar system, and by assisting the removal of newly formed H^+ ions from the canalicular end of the H transport system, thus speeding up their formation according to the law of mass action. It seems likely that the effect of the applied p.d. on the Cl^- ions would be approximately balanced by its effect on the HCO_3^- ions travelling in the opposite direction. An opposing current would have an effect on the system opposite to that of an enhancing current. A quantitative account of the effects of applied current will be given in a subsequent paper.

An inorganic system which can produce hydrochloric acid by an electron cycle mechanism A 'model' of the oxyntic cell has been made which can carry out many of the reactions believed to occur in the living cell.

The system contains two units A and B. Unit A consists of two beakers of dilute NaCl solution (1 and 2) connected by a NaCl bridge and an inverted U tube, containing H_2 , covering two bent Pt black electrodes. A stream of CO_2 bubbles through solution 1. The pH of each solution is measured with glass electrodes and the whole unit is placed on a small trolley which can move to and fro and, by producing waves, cause the NaCl solutions and the H_2 to cover the Pt electrodes alternately. Unit B consists of two beakers, one of FeCl_2 (3) and one of FeCl_3 (4) connected by an inverted U tube packed with the chloride of an anion exchange resin (De-acidite B) which can pass a

current consisting virtually of Cl^- ions only. Pt black electrodes connect the solutions with those of unit A. A battery can be connected across unit B.

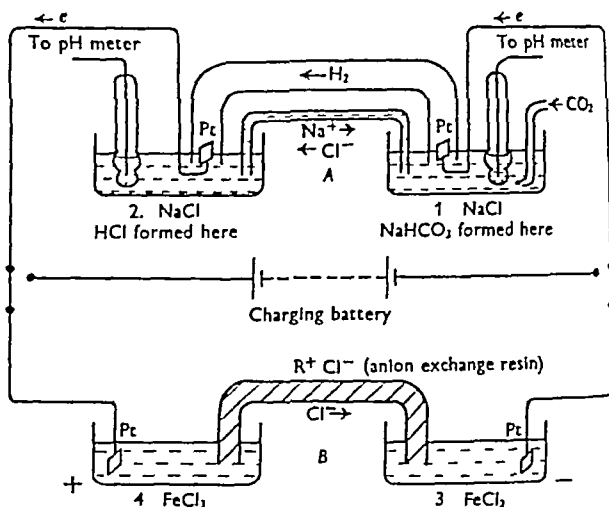
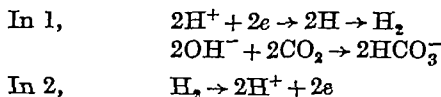
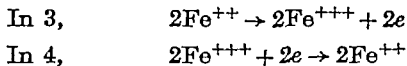


Fig. 12. Diagram of an inorganic system which can produce HCl by an electron cycle mechanism.

The reactions in unit A are

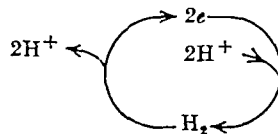


Na^+ ions migrate from 2 to 1 and Cl^- ions from 1 to 2. In unit B the reactions are



Cl^- ions migrate from 4 to 3.

The fundamental overall electron cycle is thus



In this model the reactions stop when the p.d. due to the pH differences in unit A counterbalances the redox potential of unit B. It seems probable that in the oxyntic cell this process is driven by the differences in hydrogen atom concentration maintained across the secreting unit. It is technically easier in this model to drive the process by the 'electron pressure' difference across the $\text{Fe}^{++}\text{-Fe}^{+++}$ system which can be recharged with a battery. The maximum concentration of hydrochloric acid obtainable by passing current through unit A is about twice the concentration of the salts initially present (Na^+ ions migrate to 1, Cl^- ions to 2). Starting from physiological saline solutions, this unit can produce

acid at any concentration claimed for the primary acidity of oxyntic cell secretion (see Babkin, 1944). This is taken to support the electron cycle mechanism of hydrochloric acid production.

Phenomena accounted for by the electron cycle mechanism. This electron cycle mechanism thus fits the following experimental observations on gastric mucosa: the secretion of H^+ , Cl^- and HCO_3^- ions, the relations between Q_{HCl} , Q_{O_2} and Q_{CO_2} , and the action of histamine and of carbonic anhydrase, the p.d. across the mucosa and its sign, the decrease of p.d. and increase of resistance on secretion, and the correlation between both Q_{HCl} and resistance changes with p.d. changes, the maintenance of an external current by the mucosa, and the effect of enhancing and opposing currents on acid secretion.

SUMMARY

1 The potential difference (p.d.), resistance, electrical power production and rate of acid secretion of isolated frog gastric mucosa were measured with an apparatus which is described.

2 The secretory side of frog gastric mucosa was negative with respect to the nutrient side in an external circuit. During acid secretion the p.d. was decreased and the resistance increased.

3 Anaerobically the resistance increased and both p.d. and acid secretion were abolished.

4 The mean p.d. across mucosa from the corpus of the stomach was -30 mV, rectal mucosa -16 mV, pyloric mucosa -5 mV, oesophageal, duodenal and ileal mucosa -2 mV.

5 The rise of p.d. after mounting the gastric mucosa was probably caused by the decay of injury potentials. Experiments suggest that the fall in p.d. on secretion was not due to diffusion potentials between the solutions bathing the mucosa.

6 Unstimulated frog gastric mucosae could send a current round an external circuit, producing a maintained electrical power output of the order of $1 \mu W$ /mg dry weight without appreciable polarization. About 10% of the metabolic energy was thereby canalized into electrical energy. This is taken to support the theory that secreting mucosae produce hydrochloric acid by an electrochemical mechanism.

7 Based on the foregoing and other results, an electron cycle mechanism of acid secretion is suggested, in which H atom and electron transport systems are coupled to produce a net separation of H^+ and OH^- ions in the pericanalicular zone of the oxyntic cells. The energy required is obtained from aerobic metabolism.

We wish to thank Prof. H. A. Krebs, F.R.S., for his encouragement and interest in this work.

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The Effect of Electric Current on HCl Secretion by Isolated Frog Gastric Mucosa

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Hoffmann (1889) was one of the first workers to maintain, on the basis of animal experiments, that stomach secretion was increased by direct current. Many confused and contradictory results have since been published. Claims have been made that direct current affects the motor but not the secretory activity of the human stomach (Schawerin, 1937), the motor and secretory activity (Kirstner, Gorin stem & Rudoy, 1931), and that it has no effect at all (Goldschmidt, 1895). All this early work can be criticized as insufficiently controlled. The secondary effects due to vagal stimulation, when the currents were large enough, were not separated from any direct action on the oxyntic cells.

Recently, Rehm (1945) has shown by controlled experiments on the stomach of a live dog that current passed through the wall of a secreting stomach from mucosa to serosa decreased the rate of secretion of hydrochloric acid, but that current passed from serosa to mucosa increased the rate of acid secretion. In a non secreting stomach, acid production was not initiated by passing electric current through it in either direction.

These results became available to us during our investigation into the possibility that gastric mucosa could utilize electrical energy presented to it from external sources. Since acid secretion in frog gastric mucosa is accompanied by a change in the electrical potential difference across it, it seemed possible that the rate of secretion might be affected when this p.d. is changed by passing a current through the mucosa.

The experiments described in this paper show that currents enhancing the natural p.d. of isolated frog gastric mucosa increased the rate of acid secretion and sometimes even initiated it. Currents in the opposite direction decreased the rate of acid

secretion. The effect was specific to secretive gastric mucosa, i.e. mucosa which is capable of secreting hydrochloric acid (either spontaneously or by stimulation) whether or not it is actually secreting. (Crane & Davies, 1947). The significance of the experimental results is discussed.

A part of the present work was communicated to the Biochemical Society (Crane, Davies & Longmuir, 1946).

EXPERIMENTAL

The materials, apparatus and methods used during these experiments were similar to those previously described by Crane, Davies & Longmuir (1948). Three species of frog (*Rana temporaria temporaria* L., *R. esculenta* L. and *R. esculenta ridibunda* Pallas) and toads (*Bufo bufo bufo* L.) were used.

Frog or toad gastric mucosa was mounted in a perspex holder held between two chambers at 25°. The Q_{HCl} of the mucosa (in $\mu\text{l./mg}$ dry wt./hr) was estimated from changes in pH of the secretory solution measured *in situ*. In most experiments it was also found by changing the secretory solution and estimating the acid content of the samples thus obtained either manometrically by the addition of a bicarbonate solution, or by electrometric titration using a glass electrode and 0.01N NaOH delivered from a Conway burette.

Owing to the buffering capacity of the relatively large amount of mucus secreted by frog gastric mucosa, the HCl concentration determined from the pH of the secretory solution may be considerably less than the total concentration determined manometrically or by electrometric titration (cf. Davies & Longmuir, 1948). In cases where only a pH determination *in situ* was made the value of the acid concentration was corrected for this buffering effect.

The p.d. across the mucosa was measured with a battery operated Marconi pH meter and potentiometer Type TF 511 C, using saturated calomel electrodes and saturated KCl bridges with internal ground glass joints, which dipped

into the nutrient and secretory solutions. Current was passed through the mucosa by means of zinc-zinc acetate electrodes separated from the solutions bathing the mucosa by the agar layers previously described (Crane *et al.* 1948). The resistance was obtained from the slope of the p.d. current graph, a correction being made for the resistance of the solution.

The order of procedure was as follows: after the p.d. across the mucosa had become steady, current was passed through the mucosa, usually for periods between 0.5 and 1 hr, alternating with 0.5 hr rest periods when no current was passed. So that the natural rise and fall of the Q_{HCl} should not be misinterpreted as the result of applied currents, different directions and magnitudes of the currents were used in various orders in consecutive experiments. Errors in the determinations of the change in Q_{HCl} due to a current were in general less than ± 0.2 except at low rates of secretion, when accuracies up to $\pm 10^{-4}$ were obtainable. Currents used were usually those giving densities of 0.40 and 1.0 ma/cm² of mucosa.

RESULTS

It has previously been shown (Crane *et al.* 1948) that the secretory side of isolated frog gastric mucosa is negative with respect to the nutrient (submucosal) side, the mean p.d. across the mucosa being -30 mV. Currents passed through the membrane from nutrient to secretory side increased the magnitude of the p.d. across it and are called enhancing currents. Currents passed in the opposite direction are called opposing currents. A small opposing current decreased the p.d. across the mucosa, on increasing the opposing current the net p.d. became zero and finally increased in the opposite direction. This is shown in Fig. 1, in which the p.d. across the mucosa is plotted

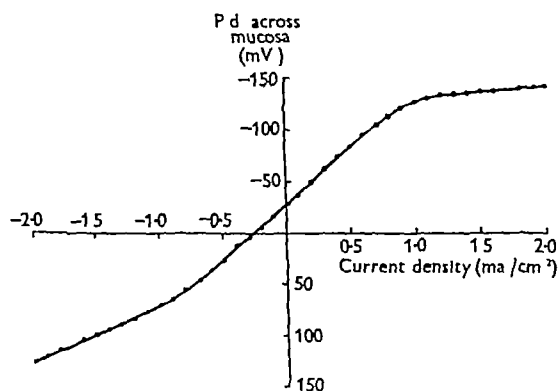


Fig. 1 Relationship between p.d. across an isolated frog gastric mucosa and current density in it. (Slope of curve at any point = resistance of 1 cm² of mucosa.)

against the applied current density for a typical frog gastric mucosa. The characteristics of this curve will be discussed elsewhere. It is shown here because it formed the basis for the choice of current densities used, 0.4 ma/cm² was usually below, and 1.0 ma/cm² usually above, the value at which the

change in slope occurred (breakdown current density). Current densities below this breakdown value produced no detectable irreversible change in the mucosa. Current densities just above the breakdown value seemed to be the maximum compatible with subsequent normal functioning of the mucosa.

Gastric mucosae from 28 frogs and 2 toads were used in these experiments, during which current was passed for eighty-four separate periods. Comparable quantitative data were obtained for sixty-one of these periods. Control experiments, which have been described elsewhere (Crane *et al.* 1948), were carried out with 150 other frog gastric mucosae through which no current was passed. Results are described for secretive mucosae when they were initially (a) secreting and (b) not secreting, for non-secretive gastric mucosae and for six non-gastric membranes.

Secretive mucosae initially secreting acid. In sixteen out of seventeen cases an enhancing current increased the rate of acid secretion from a Q_{HCl} between 0.2 and 2 during control periods to a Q_{HCl} between 1 and 4 while the current was passing. In one experiment the Q_{HCl} was reduced by 0.1 during the passage of an enhancing current of density 0.4 ma/cm². Histamine had been added to the nutrient solution in six of these seventeen cases. Fig. 2 shows the frequency distribution of the increase in Q_{HCl} , and Fig. 3 the increase plotted against the initial Q_{HCl} . There was no significant difference between the Q_{HCl} increases for the current densities of 0.4 and 1.0 ma/cm², the mean increases were 1.0 ± 0.3 and 1.2 ± 0.2 respectively. Figs. 2 and 3 also show the results for opposing currents. In six out of nine experiments the Q_{HCl} was abolished by an opposing current density of 0.4 or 1.0 ma/cm². In two out of nine experiments 1.0 ma/cm² reduced the Q_{HCl} almost but not quite to zero, in one case 0.4 ma/cm² had no apparent effect on a Q_{HCl} of 0.3. Figs. 2 and 3 show further that the absolute change in Q_{HCl} was of the same order of magnitude for both enhancing and opposing currents. The fractional change in Q_{HCl} was greater for enhancing currents, the rate of acid secretion in most cases (twelve out of seventeen) was more than doubled by an enhancing current, whereas in not one case out of nine was it reduced by more than its own value. Such a reduction would correspond to an absorption of acid or secretion of alkali. The mean fractional increase in Q_{HCl} ($\Delta Q_{HCl}/Q_{HCl}$) for the twelve experiments with an enhancing current density of 1.0 ma/cm² was 2.1 ± 0.4 and for the five experiments with 0.4 ma/cm² 1.4 ± 0.7 . Thus there is no significant difference between the two current densities. The mean fractional decrease of Q_{HCl} for the nine experiments with opposing currents was 0.8 ± 0.1 . For all seventeen experiments with enhancing currents the mean fractional increase in

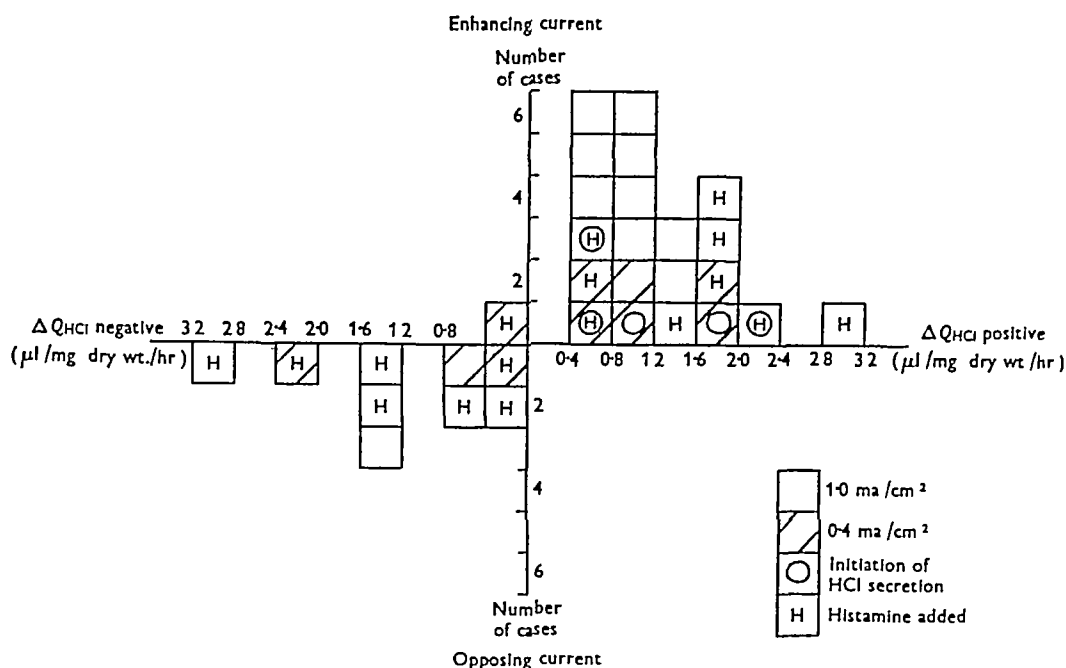


Fig 2 Frequency distribution of changes in Q_{HCl} due to currents enhancing or opposing the natural p d across isolated frog gastric mucosa

Table 1 Initiation of hydrochloric acid secretion by enhancing current

Reference number of mucosa	P d level (mV)	Experimental time			Current density (ma/cm ²)	Q_{HCl}		
		At addition of histamine (hr) (min)	At commencement of current (hr) (min)	At end of current (hr) (min)		Before current	During current	After current
145	36	—	1 4	2 29	1.0	0.0	0.4	0.0
182	37	—	1 51	2 17	0.4	0.0	1.9	0.6
141	24	1 13	2 35	3 42	1.0	0.0	2.0	2.0
229	28	1 18	3 13	4 29	0.65	0.0	0.4	0.3
229	29	1 18	7 57	9 31	1.0	0.0	0.5	0.3

Q_{HCl} was 1.9 ± 1.0 , this is barely significantly different from the value for the fractional decrease with an opposing current

Secretive mucosae not initially secreting acid Besides the experiments already discussed, Fig 3 shows five cases (four mucosae) in which the Q_{HCl} rose from zero during the passage of an enhancing current. More detailed results for these cases of apparent initiation of acid secretion by an electric current are given in Table 1. The pH changes of the secretory solution were followed continuously by means of a glass electrode and in all five cases the pH began to decrease immediately after the current was switched on. In one case (no 141) the Q_{HCl} remained unaltered when the current stopped, in three other cases the Q_{HCl} became smaller and in the remaining case acid secretion ceased when the current was switched off.

On five occasions four other secretive mucosae failed to secrete hydrochloric acid when enhancing

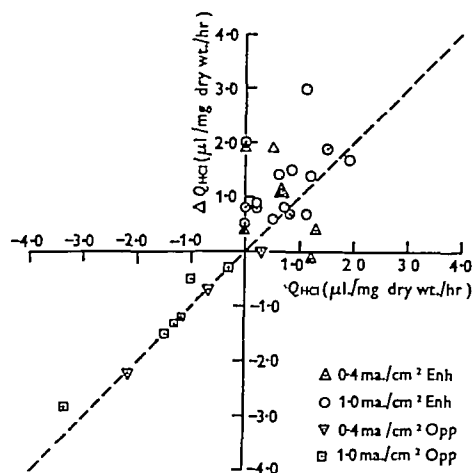


Fig 3 Relationship between Q_{HCl} and Q_{HCl} due to currents enhancing or opposing the natural p d across isolated frog gastric mucosa

current was passed through them, although they secreted acid at some other time during the experiment. Results for one of these mucosae are shown in Fig 6, in two others the current was passed before secretion commenced. One of these secreted spontaneously and the other after addition of histamine. The fourth mucosa had finished secreting before the current was passed.

Non-secretive mucosae Seven mucosae failed to secrete acid at any time during the experiments. Enhancing currents were passed through all these mucosae on sixteen occasions in all, twelve after the addition of histamine. Opposing currents were passed through these mucosae on nine other occasions, six after the addition of histamine. Since the pH of the secretory solution of a non secreting mucosa was between 6 and 7, its measurement gave a very sensitive method for detecting small hydrochloric acid concentrations. The Q_{HCl} of the above mucosae was less than 10^{-4} , the current densities were usually 0.4 or 1.0 ma/cm², but current densities up to even 50 ma/cm² failed to produce acid.

Other membranes Similar negative results with currents passed in either direction were obtained with all other membranes examined: oesophageal, pyloric, duodenal and rectal mucosa, frog skin, cellophan and dead gastric mucosa. None of these membranes (twenty three experiments) produced acid (Q_{HCl} less than 10^{-4}) and the alteration of the rate of acid secretion by an electric current thus appears to be specific to secretive gastric mucosa. It is interesting that a mucosa which secreted acid spontaneously and which responded to enhancing and opposing currents failed to secrete acid, even with an enhancing current density of 2 ma/cm², when its respiration was maximally inhibited by cyanide.

Results for individual mucosae Figs 4-8 give examples of the results for typical mucosae. Results of control experiments are shown in Figs 6-8 of another paper (Crane *et al.* 1948). Fig 4 shows results for a mucosa in which secretion began after the addition of histamine ($5 \times 10^{-5} M$) to the nutrient solution. Subsequently an opposing current density of 0.4 ma/cm², passed through the tissue from secretory to nutrient side, reduced the Q_{HCl} by 2.2. It produced a p.d. of 93 mV across the mucosa, opposite in sign to its natural p.d. of -26 mV. After a control period when acid secretion recovered slightly, an equal current passed through the mucosa in the opposite direction, enhanced the p.d. across it to -148 mV. The Q_{HCl} increased by 1.9 and returned afterwards to a lower value. The resistance of this mucosa was reduced by about half during the passage of the current, the breakdown current density was unusually low and with 0.4 ma/cm² the resistance was already below its low current value.

Fig 5 shows the effect of an enhancing current (1 ma/cm²) on a spontaneously secreting mucosa. The current was passed for three separate periods. During the first two of these the Q_{HCl} was higher than during the preceding and following control

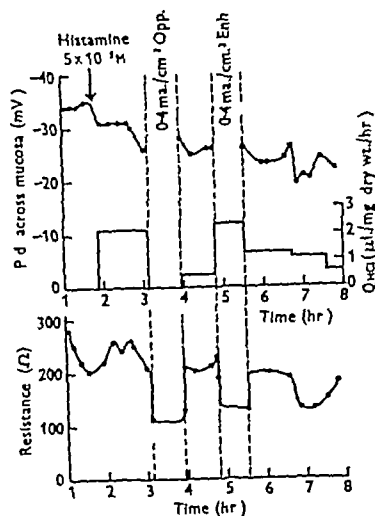


Fig 4 Effect of enhancing and opposing currents on p.d., Q_{HCl} and resistance of isolated frog gastric mucosa

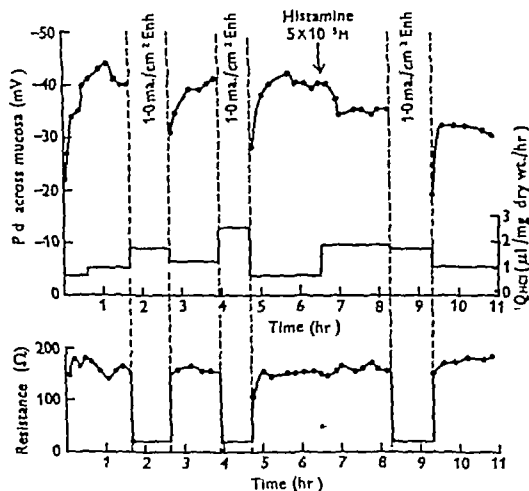


Fig 5 Effect of enhancing currents (1.0 ma/cm²) on p.d., Q_{HCl} and resistance of isolated frog gastric mucosa

periods, and the resistance only one eighth of its normal value. It is interesting that in this case a similar enhancing current after 9 hr experimental time (E.T.) had no appreciable effect, although the mucosa had responded to histamine at 6 hr 30 min E.T. Such anomalous results were observed on some other occasions many hours after mounting.

Fig 6 shows the behaviour of a mucosa which secreted for two separate periods, once spontaneously

from 2 hr 30 min to 3 hr 30 min \pm T, and again between 5 hr 30 min and 9 hr \pm T, after the addition of histamine. On three occasions during secretion an enhancing current increased the Q_{HCl} , but on two other occasions when the mucosa was not already secreting an enhancing current produced no acid whatever.

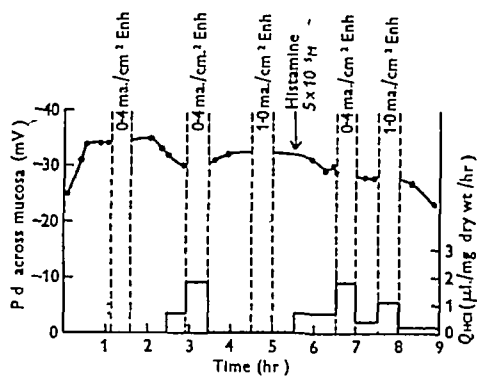


Fig 6 Effect of enhancing currents (0.4 and 1.0 ma/cm²) on p.d., and Q_{HCl} of isolated frog gastric mucosa

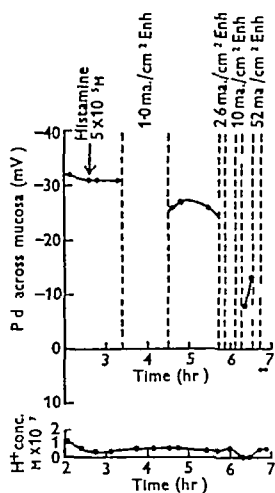


Fig 7 Effect of large enhancing currents (up to 52 ma/cm²) on p.d. across non secretive isolated frog gastric mucosa and on H^+ ion concentration of the secretory solution

Fig 7 shows results for a typical non-secretive mucosa: an enhancing current density of 1.0 ma/cm² for over an hour produced no observable effect except that the p.d. was reduced slightly. Subsequent current densities of 2.6, 10 and 52 ma/cm² also failed to produce any acid (Q_{HCl} less than 10^{-4}), but these high current densities polarized the mucosa, even reversing the sign of the subsequent p.d. in the case of 50 ma/cm². Such high currents in either direction could damage secreting mucosa so much that they became permeable to the ions present and the pH of the secretory solution rose

Fig 8 shows the results of an experiment designed to discover whether mucosa could secrete acid when a p.d. across it was maintained opposite in sign to its natural p.d. An opposing current was switched on at 1 hr 20 min \pm T and was adjusted so that the p.d. across the mucosa was 5 mV in the opposite direction to its natural p.d. of about 30 mV. The reduction of this balancing current during the next 4 hr suggests that the natural p.d. of the mucosa was

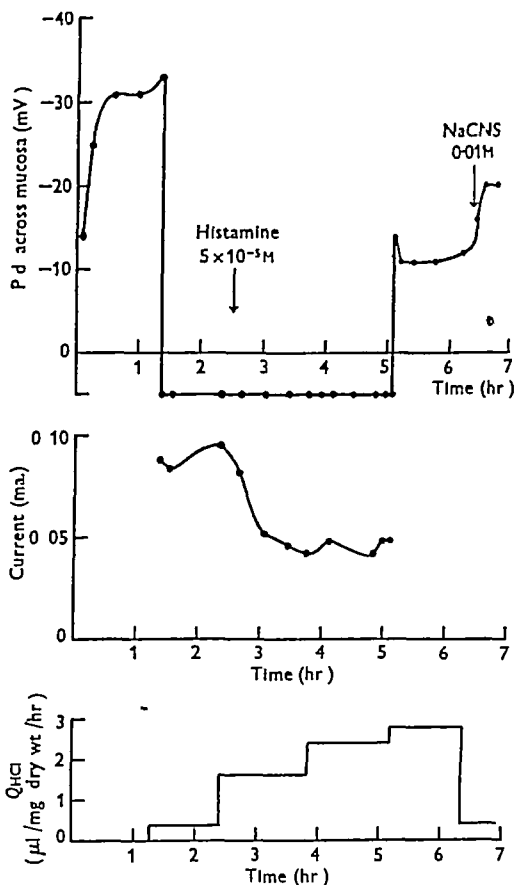


Fig 8 Secretion with p.d. across isolated frog gastric mucosa maintained at +5 mV by the application of a current. Effect of 0.01 M NaCNS on Q_{HCl} and p.d.

decreasing during this time, as has previously been found (Crane *et al* 1948). The secretion curve shows that the spontaneous secretion was increased by the addition of histamine at 2 hr 30 min and the Q_{HCl} continued to increase, rising slightly to 2.8 after the current was switched off. The p.d. was now -11 mV but rose to -20 mV on the addition of sodium thiocyanate (final concentration 0.01 M) to the nutrient solution, which reduced the Q_{HCl} to 0.4. This substantiates the previous assumption that the p.d. was reduced at the onset of secretion (cf Crane *et al* 1948).

DISCUSSION

Polarity of the effect In the experiments on human patients (e.g. Goldschmidt, 1895, Kirstner *et al* 1931, Schawerin, 1937) low-current densities were used, less than about 0.5 ma/cm^2 of electrode area. The current was passed between parallel electrodes on the dorsal and ventral surfaces of the body at the level of the stomach, so that it was impossible to investigate the effects of currents in different directions through the stomach wall. Moreover, vagal stimulation was not separated from any direct effect on the mucosa.

The experiments of Rehm (1945) on dogs, together with those on isolated frog gastric mucosae presented in this paper, establish the remarkable fact that the rate of secretion of acid by the stomach of a living animal and by isolated gastric mucosa can be altered by the passage of direct current. Since currents from the nutrient to the secretory side of the membrane enhanced the natural p.d. and increased the rate of acid secretion, whilst currents in the opposite direction opposed the natural p.d. and decreased the rate of secretion, the effects cannot be due to stimulation of nerve endings.

These two independent series of results also show that the applied currents do not alter the Q_{HCl} by emptying reservoirs of acid in the mucosa or damming back the acid. If this were the case the raised Q_{HCl} during enhancing current flow would be counterbalanced immediately afterwards by a Q_{HCl} depressed below normal. Similarly, after opposing currents, a Q_{HCl} above normal would be expected. These effects did not occur. An electroosmotic effect on the secretion of water cannot be the sole cause, since this would merely produce a more dilute secretion containing the same total amount of acid.

In general, frog gastric mucosa appeared to be little, if at all, damaged by the current densities used. Current densities of 0.4 ma/cm^2 had no effect on the subsequent p.d. of the mucosa (Fig. 4), but the p.d. was sometimes temporarily reduced for 10–30 min after current densities of 1.0 ma/cm^2 (Fig. 5). A few poor mucosae, however, never recovered their original p.d. after 1.0 ma/cm^2 . Higher current densities were more injurious, the effect of progressively increasing enhancing currents on the p.d. may be seen in Fig. 7. In general, we confirmed Rehm's results (Rehm, 1945) that high opposing currents caused more damage than similar enhancing currents. A comparison of the relative change in Q_{HCl} in Rehm's and our own experiments is interesting. The examples given by Rehm (1945) (e.g. his Figs. 2, 3A, 3B, 4A) suggest that for 1 ma/cm^2 an enhancing current increased the Q_{HCl} by c. 20% and the same opposing current decreased it by c. 60%, whereas in the present experiments the

average increase was 210% and the average decrease 80% with the same current densities. The gastric mucosa of the dogs used by Rehm (1945) had Q_{HCl} values up to about 50, whilst the isolated frog mucosa used in these experiments had Q_{HCl} values up to about 4.

In Rehm's (1945) experiments the change in Q_{HCl} was found to be roughly proportional to the current used ($0.4\text{--}4 \text{ ma/cm}^2$) for both enhancing and opposing currents, the latter being about three times as effective as the former. The present experiments give information only for current densities of 0.4 and 1.0 ma/cm^2 , but for these the absolute change in Q_{HCl} was not very different, and while the fractional change in Q_{HCl} was 1.5 times as great for the higher as for the lower current density, the difference was not significant. However, the power consumed at these two current densities may be similar. It seems possible that there is a real difference in the ΔQ_{HCl} current relationships of dog and frog gastric mucosa, but more results are needed.

Specificity The increased production of acid when enhancing current is passed through secretive gastric mucosa is extremely specific. In twenty one out of twenty two cases enhancing current increased the Q_{HCl} of gastric mucosae already secreting acid. In the remaining case ΔQ_{HCl} was -0.1 ± 0.2 , but this is not significantly negative.

It is interesting that while in five out of ten cases enhancing current produced no acid in secretive mucosae not already secreting, in the other five it initiated acid secretion. It is extremely unlikely that spontaneous acid secretion should have chanced to begin in all cases just as the current was switched on (see Table 1). Rehm (1945) found no initiation of acid secretion by enhancing current (density up to 7 ma/cm^2) in any of sixteen cases with live dogs. However, since 'spontaneous' secretion of acid can occur in isolated frog gastric mucosa, but does not occur in the stomachs of live dogs, this difference of behaviour is perhaps not surprising.

The absence of any acid production when current was passed on twenty five occasions through non secretive gastric mucosae and on twenty three occasions through oesophageal, pyloric, duodenal, rectal mucosae, frog skin, cellophan and dead gastric mucosa, strongly suggests that the effect is specific to secretive gastric mucosae, and shows that it is not due to a simple electrolysis. This is confirmed by the absence of any evidence of alkali secretion due to the passage of a current through gastric mucosae or any of the other membranes used.

Significance of the results Nearly 90 years ago Brücke (1859) suggested that the electromotive forces in gastric mucosa may enable it to send an acid fluid towards the secretory surface and a basic fluid in the opposite direction. Since then many workers (e.g. Engelmann, 1872, Boenheim, 1930,

Keller, 1932) have suggested that glands produce their secretions by electroendosmosis. An attempt was made by Mudd (1926) to prove this hypothesis by applying an electric field, and he believed his data on mammalian serous membranes 'to indicate a probability approaching certainty that electroendosmotic effects are a factor in glandular secretion'.

The first controlled experiments were carried out by Rehm (1945) on the stomachs of live dogs, he has produced much other evidence with this tissue in support of the hypothesis that 'electrical energy provides the energy for the production of osmotic work by living cells' (Rehm, 1943, 1944, 1946, Rehm & Enelow, 1945, Rehm & Hokin, 1947). This work has been confirmed and extended by experiments on isolated frog gastric mucosa (Crane *et al.* 1946, 1948, Crane & Davies, 1947, Davies, Longmuir & Crane, 1947).

It has been shown (Davies, 1948) that, however complex the intermediary processes, the H^+ ions which are elaborated by oxyntic cells come initially from the H atoms in water. In this process electrical work must be done to separate the charges. The experiments of Rehm *et al.* and the present writers, referred to above, together with those recorded in this paper, make it difficult to avoid the conclusion that secreting gastric mucosa can use electric energy from an external source in the production of acid.

Interpretation of the effect. An electron cycle mechanism of acid secretion has been proposed (Crane *et al.* 1948), in which H atom and electron transport systems are coupled to produce a net

separation of H^+ and OH^- ions in the pericanalicular zone of the oxyntic cells. On the basis of the electron cycle mechanism, the electrical power available from the applied currents and from metabolism will be compared, in a forthcoming paper, with the power needed to produce the observed values of the Q_{HCl} .

SUMMARY

1 Currents up to 50 ma/cm² were passed through isolated frog gastric mucosa, in directions both enhancing and opposing the natural p d.

2 In gastric mucosae secreting acid, enhancing currents (from nutrient to secretory side) increased the Q_{HCl} for 0.4 ma/cm², mean $\Delta Q_{HCl} = 1.0 \pm 0.3$, for 1.0 ma/cm², mean $\Delta Q_{HCl} = 1.2 \pm 0.2$. Similar opposing currents decreased the Q_{HCl} by the same order of magnitude, in many cases secretion was stopped.

3 Four secretive gastric mucosae, not initially secreting acid, commenced to do so when an enhancing current was passed through them, in four other secretive gastric mucosae acid secretion was not initiated by enhancing currents.

4 In all cases currents passed through non-secretive gastric mucosa failed to initiate acid secretion.

5 No other membranes examined (oesophageal, pyloric, duodenal and rectal mucosae, frog skin, cellophan and dead gastric mucosa) produced hydrochloric acid with any currents applied.

We wish to thank Prof H. A. Krebs, F.R.S., for his encouragement and interest in this work.

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Studies in the Nitrogen Metabolism of the Apple Fruit

CHANGES IN THE NITROGEN METABOLISM OF THE APPLE DURING THE NORMAL AND ETHYLENE-INDUCED CLIMACTERIC RISE IN RATE OF RESPIRATION

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During work carried out over a number of years, it has been found repeatedly that, in Bramley's Seedling apples which have been gathered before the onset of the respiration climacteric and placed in storage, there is a slow rise in net protein content during the period when the respiration climacteric occurs (see, e.g., Hulme, 1936b). It is not certain, however, that there is a direct connexion between these two phenomena, it may be that both are changes normally occurring in ripening fruit and that their concurrence may be fortuitous.

Kidd & West (1932) have demonstrated that the respiration climacteric can be induced even in immature fruits by treatment with ethylene. A net rise in protein content, therefore, in relatively immature apples during an ethylene induced climacteric rise in respiration would be strong presumptive evidence that the two phenomena are intimately connected. The experiments described in this paper were designed, primarily, to ascertain whether this is the case. Data are also given for changes occurring during the 'normal' (i.e. not ethylene-induced) climacteric rise in respiration which occurs eventually even in immature fruits.

Data on the nitrogen metabolism of apples at various stages of development on and off the tree, which have accumulated in this laboratory over a number of years, have suggested that, of the soluble nitrogen fractions presumably involved in protein synthesis, asparagine nitrogen is most closely associated with rise or fall in protein. The present experiments provided an opportunity for studying such a relationship over a short specialized period in the ontogeny of the fruit. The period of the climacteric rise in respiration in immature fruits is especially suitable for such a study, since it embraces a relatively rapid rise in protein under conditions of constant content of total nitrogen.

EXPERIMENTAL

Materials and methods of sampling and storage

Bramley's Seedling apples at two stages of maturity were selected for the experiment. They were taken from 18 trees (18 years old) on East Malling stocks I and II on an unmanured plot at East Malling Research Station. The date

of petal fall was 17 May 1937, and the two series of samples were picked on 17 June (Series 1) and 3 August (Series 2). At each pick the requisite number of apples of uniform size was gathered, and each sample was made up from a definite number of fruits from each tree. This procedure ensures that the samples are uniform, each being representative of the group of trees as a whole. Series 1 consisted of sixteen samples, each of 22 fruits, the average weight per apple was 160 g. Series 2 consisted of twenty-one samples, each of 30 fruits, the average weight per apple was 102.5 g. Both series of fruits were, therefore, very immature, the normal commercial date of picking for this variety being about the end of September.

Immediately after preparation, three or four samples were rapidly halved, the seeds removed and the fruits of each sample placed in tins and frozen to -20° to await analysis (Hulme, 1936a). These fruits are the 'initial' samples. The remaining samples were each placed in their respective respiration chambers, which formed part of an apparatus for the determination of their rate of respiration in presence and absence of ethylene. This train of apparatus, which was situated in a constant-temperature room at 12° , was similar to that already described (Hulme, 1937), except that a T piece was inserted between the KOH bubbler and the respiration chamber so that ethylene could be introduced into the train at a definite rate through a needle valve and a calibrated bubble bottle. In the case of Series 1 the respiration chamber took the form of a 2 l. Buchner flask in place of the usual vacuum desiccator, and the CO_2 absorption towers were replaced by Pettenkofer tubes.

Preparation of material for analysis and methods of analysis

Separation into peel and pulp tissue and disintegration of the separated tissues was carried out as already described (Hulme, 1936a). The seeds of the fruits were collected and their total N content determined so that an indication could be obtained of any possible migration of N between flesh and seed. This factor must be considered, since, in the immature fruits used in these experiments, the seeds were only partially developed and appeared to be still organically connected to the cortical tissue.

Total N was determined on a sample of frozen, ground tissue by the usual Kjeldahl method using 100% (v/v) H_2O_2 as catalyst in addition to $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$.

The various N fractions* were estimated as previously described (Hulme, 1936a), the rapid continuous vacuum

* It has been shown (Hulme, 1936b) that glutamine is absent from the apple fruit.

extractor (Hulme & Roach, 1936) being used for the 85% ethanol extraction of the tissue

Titrateable acid was determined in the pulp tissue by the method used previously (Hulme, 1936a)

Dry weight was determined by drying a sample of frozen ground tissue *in vacuo* at 60° for 48 hr. The results agreed closely with those obtained by exhaustive drying *in vacuo* at 25° over P₂O₅.

Statistical treatment Replicate samples were taken at intervals as follows. *Series 1* Four initial samples, two samples at the 15 days' storage point. *Series 2* Three initial samples, two at 27 days, two at 35 days and two at 55 days. Each series was treated as a separate entity in the statistical analysis of the results. Standard deviations, covering total errors, were calculated from the following equations (Fisher, 1928) $\text{Variance} = \Sigma \delta^2 / \text{degrees of freedom}$, δ being the deviation of replicates from their mean. Standard deviation, $\sigma = \sqrt{\text{variance}}$. Statistical analysis of the respiration data was considered unnecessary since determinations were made at very frequent intervals and replicate results agreed to within 2–3 mg CO₂/100 g/100 hr, even during the period of rapid change associated with the characteristic rise in respiration.

Ethylene treatment

Series 1 The dosage of ethylene in all cases was c. 1.5% in the air passing over the treated fruits. This relatively

high concentration was used to obtain an immediate response to the introduction of the gas. All twelve samples had been respiring in pure air at 12° for 28 days when the first sample was treated with ethylene, several samples were treated at 36 days, and one final sample at 46 days from zero storage time. Some samples were never treated with ethylene. The time of treatment with ethylene and the time at which treated and untreated samples were frozen to await analysis will be seen from Fig. 1. Where several samples were treated at the same time, the subsequent rate of respiration shown in Fig. 1 is the mean rate of all these samples.

Series 2 Dosage was approximately the same as for Series 1, but all the samples to be treated were given ethylene at the same time, i.e. 20 days after placing in the respiration chambers. The points at which treated and untreated samples were frozen to await analysis are indicated in Fig. 2. The rates of respiration subsequent to ethylene dosage for both treated and untreated samples are the means of the rates for the samples remaining under observation. The number of samples represented by the rate shown in Fig. 2 decreased as samples were removed and frozen.

During the course of the experiments one or two fruits in some of the samples developed infection by moulds. As soon as this was noted the fruit concerned was removed and weighed and the necessary correction applied to the subsequent calculations of rate of respiration.

Table 1. *Apple weight and nitrogen content (per apple) of ethylene-treated and untreated samples, Series 1, stored at 12°, pulp and peel tissue*

Days in store	Ethylene treatment (+ or -)	Initial fresh wt of whole fruit (g)	Dry wt (mg)	Total N (mg)	Protein N (mg)	Ammonia N (mg)	Asparagine N (mg)	Amino acid N (mg)	Residual N (mg)	Titrateable acid* (mg)
Pulp										
0	-	15.9	1393	12.35	8.70	0.05	2.32	0.32	0.96	256
3	-	17.0	1460	13.72	8.87	0.05	2.96	0.62	1.22	278
7	-	16.1	1338	12.80	7.68	0.05	3.08	1.05	0.93	261
15	-	15.6	1293	13.33	6.55	0.09	4.37	0.81	1.52	238
28	-	15.5	1237	12.50	6.15	0.12	3.92	0.68	1.61	209
36	+	16.2	1248	12.53	7.48	0.09	3.12	0.35	1.49	195
36	-	16.0	1143	12.78	6.80	0.13	3.90	0.54	1.43	199
38	+	15.9	1212	13.80	7.20	0.12	4.40	0.46	1.62	198
41	+	16.1	1164	12.54	7.51	0.11	3.31	0.38	1.23	190
46	+	16.3	1191	13.15	8.11	0.11	3.12	0.52	1.29	183
46	-	16.8	1256	14.09	7.47	0.17	4.26	0.46	1.71	184
64	+	15.3	1024	12.73	8.54	0.11	2.42	0.28	1.18	132
σ		1.13	93	0.64	0.40	0.017	0.25	0.058	0.11	21
Peel										
0	-	15.9		4.67	4.14		0.27			
3	-	17.0		5.03	4.30		0.33			
7	-	16.1		4.37	3.47		0.44			
15	-	15.6		4.48	3.19		0.76			
28	-	15.5		4.30	3.00		0.87			
36	+	16.2		4.50	3.27		0.84			
36	-	16.0		4.32	2.79		1.04			
38	+	15.9		4.69	3.12		1.12			
41	+	16.1		4.61	3.11		1.08			
46	+	16.3		4.87	3.50		1.01			
46	-	16.8		4.81	3.19		1.06			
64	+	15.3		4.27	3.15		0.81			
σ				0.39	0.32		0.049			

* Expressed as malic acid

RESULTS

The primary data for the fruit used and the content of the various nitrogen fractions of the fruit and the total nitrogen content of the seeds during storage with and without ethylene treatment are shown in Tables 1-3. Owing to the small amount of material available, it was only possible to determine total, protein and asparagine+ammonia nitrogen in the peel of the fruit of Series 1.

The levels of significance as between two results in each series of samples (the 'significant difference'), calculated on the basis of a 20% probability ($P=0.05$) are $\sigma \times 3.17$ for Series 1 and $\sigma \times 2.94$ for Series 2. These values may be approximated to $\sigma \times 3$, so that in both series a difference between two results greater

than three times the appropriate standard deviation may be regarded as significant.

An examination of the appropriate significant differences shows that in both series there is no real variation, due to uneven picking, in the weight of the average apple throughout the series and that, as would be expected, there is no significant change in the total nitrogen content during storage. Changes in the fractions of nitrogen are therefore more readily apparent when expressed as a percentage of total nitrogen. The standard deviations for the various nitrogen fractions expressed on this basis are shown in Table 4.

The changes in protein nitrogen, as a percentage of total nitrogen, together with the corresponding changes in rate of respiration are shown in

Table 2 *Apple weight and nitrogen content (per apple) of ethylene treated and untreated samples, Series 2, stored at 12°, whole fruit (obtained from separate results for pulp and peel) and peel tissue*

Days in store	Ethylene treatment (+ or -)	Initial fresh wt of whole fruit (g)	Dry wt (g)	Total N (mg)	Protein N (mg)	Ammonia N (mg)	Asparagine N (mg)	Amino acid N (mg)	Residual N (mg)	Titratable acid* (mg)
Whole fruit										
0	-	103.0	14.12	53.62	32.25	0.10	12.17	4.35	4.76	1603
3	-	105.4	14.93	52.00	31.40	0.13	10.92	3.92	5.62	1616
8	-	103.6	14.54	52.68	33.54	0.10	10.07	3.81	5.26	1559
20	-	102.3	13.95	51.96	32.93	0.13	10.02	3.40	5.49	1513
21	+	101.6	13.66	51.50	32.27	0.14	9.50	4.06	5.52	1532
23	+	104.7	13.81	50.18	33.00	0.11	8.75	3.68	4.64	1576
27	+	102.1	13.35	52.39	34.41	0.10	9.54	3.33	5.00	1504
27	-	102.7	13.65	52.22	31.67	0.14	11.72	3.75	4.84	1545
29	-	102.0	13.32	50.85	32.11	0.16	9.94	3.18	5.41	1541
35	-	103.0	13.68	54.27	33.48	0.20	11.98	2.69	5.86	1501
38	-	102.4	13.34	51.50	34.69	0.19	9.44	2.83	4.36	1485
38	+	103.3	13.27	50.54	35.30	0.14	8.05	2.62	4.44	1400
41	-	103.0	13.20	53.94	35.37	0.19	10.92	2.41	5.05	1464
55	-	102.4	13.08	52.69	37.20	0.24	8.54	1.81	4.93	1383
56	+	101.3	12.62	52.66	38.03	0.33	7.81	1.79	4.62	1291
70	-	104.0	12.66	50.63	37.46	0.43	6.40	2.20	4.14	1239
σ	.	1.50	0.34	2.06	1.37	0.017	0.75	0.33	0.39	30
Peel										
0	-	103.0	2.18	11.96	10.06	0.02	0.83	0.54	0.51	
3	-	105.4	2.17	11.99	10.22	0.03	0.62	0.50	0.62	
8	-	103.6	2.32	12.95	10.93	0.02	0.69	0.63	0.68	
20	-	102.3	2.15	11.96	10.04	0.03	0.66	0.47	0.76	
21	+	101.6	2.12	11.48	9.63	0.03	0.68	0.44	0.71	
23	+	104.7	2.09	11.08	9.46	0.02	0.55	0.45	0.60	
27	+	102.1	2.12	11.89	10.22	0.02	0.63	0.45	0.57	
27	-	102.7	2.09	11.07	9.04	0.03	0.76	0.43	0.71	
29	-	102.0	2.09	11.10	9.31	0.03	0.70	0.47	0.59	
35	-	103.0	2.07	11.33	9.48	0.03	0.77	0.36	0.69	
38	-	102.4	2.10	11.31	9.83	0.02	0.66	0.43	0.46	
38	+	103.3	2.06	11.54	10.30	0.03	0.41	0.23	0.57	
41	-	103.0	2.08	11.67	10.08	0.02	0.66	0.28	0.62	
55	-	102.4	2.06	11.99	10.79	0.03	0.37	0.23	0.56	
56	+	101.3	2.01	12.14	10.93	0.03	0.35	0.21	0.62	
70	-	104.0	2.00	12.03	10.90	0.04	0.30	0.31	0.48	
σ			0.030	0.41	0.40	0.005	0.049	0.033	0.052	

* Expressed as malic acid.

Table 3 *The number, weight and nitrogen content of the seeds of treated and untreated fruits of Series 1 and 2*

Days in store	Ethylene treatment (+ or -)	No of seeds per apple	Average wt per apple (mg)	Average wt per seed (mg)	Total N	
					Per apple (mg)	Per seed (mg)
Series 1						
0	-	50	117	23	0.57	0.113
3	-	45	110	25	0.53	0.120
7	-	46	117	25	0.57	0.122
15	-	45	96	21	0.44	0.097
28	-	44	89	20	0.33	0.075
36	+	49	74	15	0.25	0.050
36	-	49	72	15	0.23	0.052
38	+	51	73	14	0.26	0.049
41	+	41	60	14	0.19	0.044
46	+	45	63	14	0.21	0.044
46	-	42	65	15	0.23	0.051
64	+	42	45	11	0.24	0.050
σ		0.33	10	2.2	0.046	0.0136
Series 2						
0	-	49	194	40	1.77	0.364
3	-	44	181	41	1.69	0.385
8	-	48	183	38	1.85	0.385
20	-	40	128	33	1.37	0.352
21	+	47	166	35	1.70	0.361
23	+	48	162	34	1.84	0.383
27	+	46	154	33	1.79	0.391
27	-	49	144	30	1.59	0.326
29	-	45	136	30	1.47	0.328
35	-	46	128	28	1.33	0.293
38	-	41	123	30	1.57	0.386
38	+	52	139	27	1.57	0.300
41	-	49	133	27	1.50	0.305
55	-	43	121	28	1.57	0.366
56	+	43	114	26	1.54	0.355
70	-	.				
σ	.	0.51	13	1.7	0.147	0.0319

Figs 1 and 2 With the treated samples the rate of respiration immediately before introduction of ethylene does not fall exactly on the line representing the mean respiration of all untreated samples (continuous line)

Changes in protein in relation to changes in rate of respiration In view of the fact that there is no significant change in the absolute amount of total nitrogen in the fruit during storage, the results presented in Figs 1 and 2 indicate clearly that, over the period of the respiration climacteric, whether ethylene induced or whether occurring as a normal phenomenon in the ontogeny of the fruit, the net amount of protein in both peel and pulp rises significantly. In Series 1 the significant difference for protein nitrogen as a percentage of the total nitrogen is 4.2 for whole fruit and 4.5 for the peel. In all cases the rise in protein nitrogen in the whole apple following ethylene treatment is more than twice the significant difference. In the peel, all the rises are significant except that after the first ethylene treatment, even in this case there is no indication that the rise has ceased when the only treated sample was frozen for analysis. In Series 2,

where the significant differences are 2.2 and 2.0% respectively, the rise in protein nitrogen subsequent to ethylene dosage is highly significant for both whole fruit and peel. In this series also the onset of the normal climacteric is followed by a highly significant rise in protein nitrogen in both whole fruit and peel.

It appears, however, that in both peel and pulp tissues of both series the rise in respiration precedes by some hours the rise in protein content (Fig 1, ethylene treatment at 36 hr and Fig 2, both ethylene treated and untreated samples). The immediate rise in protein following ethylene treatment at 36 hr in the peel of the fruit of Series 1 is not significant. It is interesting to note that in experiments designed to determine the relative rate of respiration of peel and pulp tissue it has been found (Hulme, unpublished data) that the peel of fruits of 100 g average weight respire at least three times as rapidly as the pulp tissue. As the present data show, the protein content per unit dry weight of peel tissue is at least twice that of pulp tissue and forms a higher percentage of the total nitrogen (see also Hulme & Smith, 1938).

Changes in the nitrogen content of the seeds It is clear that the average number of seeds per apple does not vary significantly throughout all the samples (Table 3) The total nitrogen of the seeds

Table 4 *Standard deviations for various nitrogen fractions expressed as a percentage of total nitrogen*

Tissue and fractions		Standard deviation (σ)
Series 1		
Whole apple		
Protein N		1.39
Asparagine + ammonia N		1.04
Pulp		
Protein N		1.78
Ammonia N		0.13
Asparagine N		1.23
Amino acid N		0.37
Residual N		0.37
Peel		
Protein N		1.53
Asparagine + ammonia N		0.30
Series 2		
Whole apple		
Protein N		0.73
Ammonia N		0.021
Asparagine N		0.97
Amino acid N		0.81
Residual N		0.40
Peel		
Protein N		0.67
Ammonia N		0.053
Asparagine N		0.33
Amino acid N		0.33
Residual N		0.44

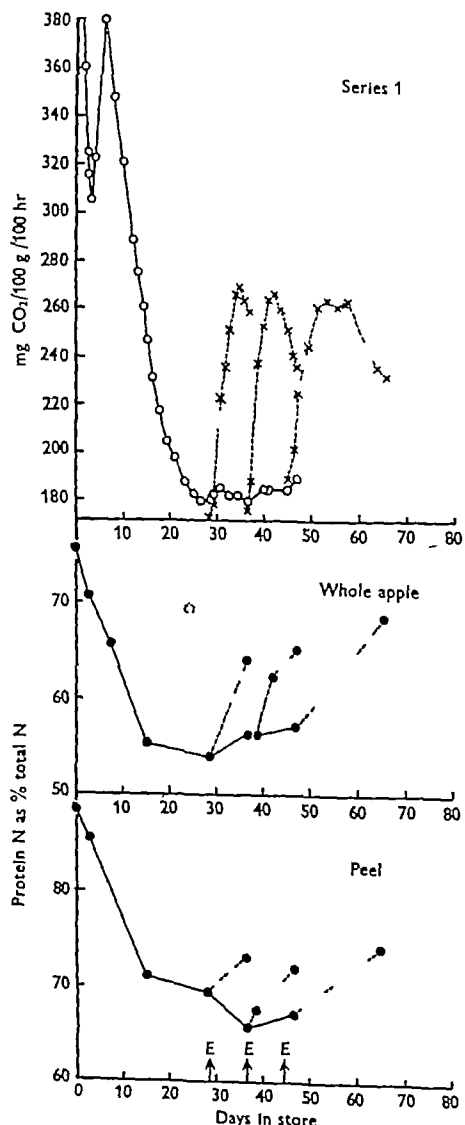


Fig 1 The change in the rate of respiration (top graph) and the change in content of protein N in the whole apple and in the peel during the storage of apples of Series 1 in air and in ethylene An E above an arrow indicates the point at which ethylene was given to samples whose subsequent history is indicated by an interrupted line

per apple falls rapidly in Series 1 during the first 36 days of storage, but there is no evidence of any change during the climacteric rise in respiration whether ethylene induced or not The fate of the nitrogen lost in the early stages of storage is not

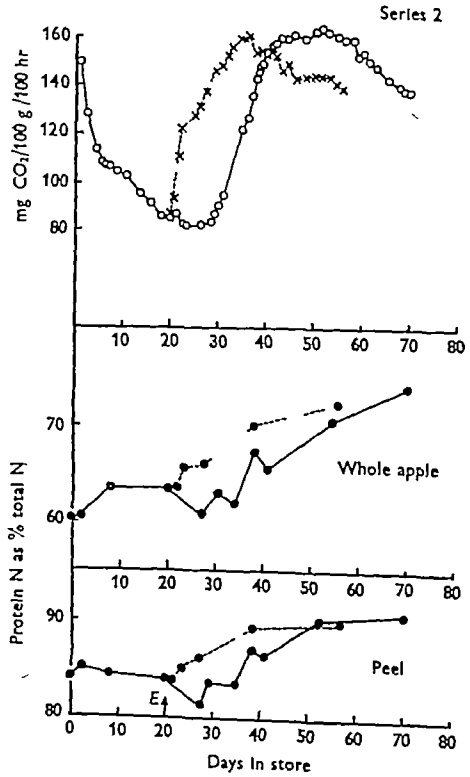


Fig 2 Changes similar to those depicted in Fig 1, but for the fruit of Series 2

clear. In Series 2 there is no significant change in the nitrogen content of the seeds at any time. Thus no transfer of nitrogen from seeds to flesh tissue occurs at any time during the storage of the fruit.

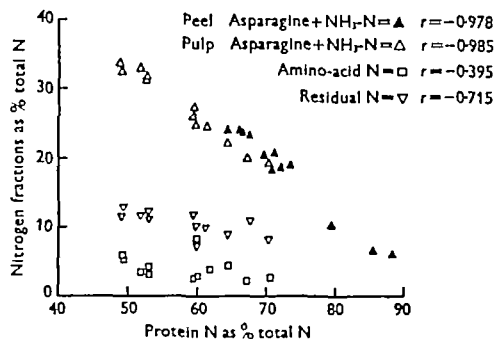


Fig 3 Relation between protein N and the non protein N fractions over the whole period of storage of Series 1 fruit, both normal and ethylene treated

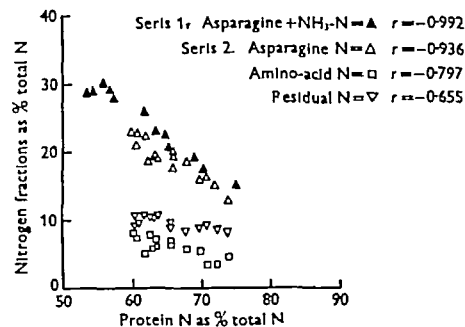


Fig 4 Relation between protein N and the non protein N fractions over the whole period of storage of Series 1 and 2 fruits, both untreated and ethylene treated. The results shown are for the whole fruit.

Changes in the fractions of the non-protein nitrogen during increase or decrease in protein. Since total nitrogen is constant, it may be expected that all the forms of non-protein nitrogen will be affected to some extent by change in content of protein. A cursory examination of the data presented in Tables 1 and 2 suggests that the changes in asparagine nitrogen are most closely related to rise and fall in protein. The relationship between protein nitrogen and the non-protein nitrogen fractions is shown in Figs 3 and 4. The close correlation between rise in protein nitrogen and fall in asparagine nitrogen is striking. Furthermore, the change in asparagine nitrogen per unit protein (or vice versa) is the same for peel and pulp and covers a wide range of protein levels. The values for asparagine nitrogen in Fig 4, Series 1, are rather high compared with those of Series 2 owing to the inclusion of ammonia nitrogen in the former case. It is clear that, at both stages of

maturity examined, a similar relationship exists throughout the fruit between fall in asparagine and rise in protein.

This general close relationship is confirmed on examination of the correlation coefficients for protein nitrogen on asparagine plus ammonia nitrogen, amino acid nitrogen and residual nitrogen. Asparagine gives a coefficient (r) of almost unity in every case. The correlation coefficients for amino acid nitrogen and residual nitrogen are also fairly high, the former not consistently so. Little is yet known of the composition of the residual nitrogen, although it is certain that basic amino-acids are present only in small amounts in the apple (Hulme, 1937).

Titratable acid. The determination of titratable acid has been carried out as a matter of routine in the study of the nitrogen metabolism of apples which has been proceeding in this laboratory for some years. The results obtained in the present study are included in the tables, since it is clear that immediately before the onset of the respiration climacteric (normal and ethylene induced) there is a rise in the amount of titratable acid in these immature fruits. This rise is, however, only significant in the case of the samples of Series 2.

DISCUSSION

The above data are insufficient for the development of a theory of the paths of protein synthesis in the apple fruit, the most that can be said is that deamidization of asparagine appears to be closely connected with the synthetic processes. Of interest in this connexion is the fact that, while free ammonia nitrogen never forms much more than 1% of the total nitrogen, it does tend to rise significantly during active protein loss and when the rate of protein synthesis is slowing down (Series 1 at day 28, Series 2 towards the end of the storage period).

SUMMARY

1 Evidence is produced that during the climacteric rise in rate of respiration in immature apples, whether occurring normally or whether induced by treatment of the fruit with ethylene, there is a highly significant rise in the net protein content of the fruit.

2 A strong negative correlation is shown to exist between change in protein content and change in asparagine content, not only during the rise in protein which accompanies the respiration climacteric, but also during the steady fall in protein content which precedes the respiration climacteric in very young fruits. The other forms of soluble nitrogenous constituents show some negative correlation with change in protein content, but the correlation is less consistent.

I wish to make grateful acknowledgement to Mr J Taylor of East Malling Research Station for his assistance with the statistical analysis involved in this paper Mr W Arthington assisted in carrying out the experimental

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The Algae

4 THE LIPOCHROMES OF THE MALE AND FEMALE GAMETES OF SOME SPECIES OF THE FUCACEAE

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Biochemical work done hitherto on the algae has been concerned chiefly with the vegetative thallus. Mainly because of the difficulties of securing pure material in sufficiently large quantities, very little consideration has until recently been given to the pigmentation of the reproductive bodies. Among the lower plants, the male gametes of many algae are known to be yellowish in colour, in contrast to the rather more green female gametes, and this difference is especially marked in heterogamous and oogamous plants.

The members of the family Fucaceae (class Phaeophyceae), so abundant on many parts of our British shores, reproduce oogamously by means of minute motile, biciliate sperms and larger non-motile eggs. The dioecious members of the Fucales are especially convenient for study, in that large quantities of both male and female gametes can be obtained in a pure state and free from any possibility of contamination by gametes of the other sex. In the same family, monoecious species also occur, and thus provide material for comparison with the dioecious types.

In the Fucaceae, the antheridia and oogonia are produced within flask shaped, sunken cavities, the conceptacles. These occur at the periphery of somewhat swollen and mucilaginous receptacles, which in some species are modifications of some of the dichotomies of the thallus as in *Fucus serratus*, *F. vesiculosus* and *F. spiralis*, or in other genera, of the apices of special lateral deciduous fruiting bodies, as in *Ascophyllium nodosum*.

The gradual extrusion of groups of mature eggs and sperms through the openings (ostioles) of the conceptacles, normally occurs between the tides, and is probably brought about in part by the expansion of mucilage, together with contraction on desiccation. In nature, the gametes are removed from the surface of the conceptacles by the incoming tide and fertilization thus takes place outside the parent plant.

The numerous minute biciliated sperms contain a small yellowish chromoplast, and the mature aggregates of sperms therefore appear orange yellow, which colour they impart to the entire receptacle. This affords a means of distinguishing the male plants in the dioecious species of the Fucales at the time of gamete discharge. In the preparation of material for the present experiments, however, colour alone was not considered to be a sufficiently safe criterion, and a section of a receptacle of every plant collected was examined microscopically, in order to confirm the accuracy of the separation of the male and female plants. Moreover, plants were selected from all parts of the zone of their distribution on the Aberystwyth shore.

The marked orange colour of the exudate of the male plants of the dioecious members of the Fucaceae in their fruiting season, in striking contrast to the olive green colour of the female plant exudate, suggested that the colouring matter of the chromatophores of the male and female gametes might possibly be connected with the reproductive process. It seemed feasible that the light absorbing

properties of the pigments might be responsible for the motility of the male gametes prior to fertilization of the female egg (oosphere)

Immersion in water of the receptacles of the fruiting male plants collected at low tide produces a bright orange suspension of sperms and antheridia, whilst from the fruiting female plants a suspension of eggs (and oogonia) and copious mucilage is secured. From the fruiting monoecious plants, a mixture of eggs and sperms is obtained, the proportions of each varying somewhat during the fruiting period. In the earlier experiments on *Fucus serratus* the orange exudate was removed by wiping, but this method was later rejected in favour of dipping the receptacles in water. From these suspensions the pigments can be extracted with ether or light petroleum, or better still the suspension can be concentrated by centrifuging or filtration. The suspension obtained in sea water consists almost exclusively of active free-swimming sperms with empty antheridial vesicles from which the sperms have been released. A suspension in fresh or distilled water, on the other hand, consists mainly of antheridia with undischarged gametes which renders the process of concentration easier. From the male exudate an orange solution is thus obtained, containing a pigment which exhibits the normal properties of a carotenoid hydrocarbon or ester, i.e. solubility relationships, absorption spectrum, coloration with antimony trichloride in chloroform solution and adsorption properties on alumina, calcium hydroxide and magnesium oxide.

It has been established that the characteristic carotenoid pigments of fresh brown algae are β -carotene and fucoxanthin (Heilbron, Parry & Phipers, 1935, Carter, Heilbron & Lythgoe, 1939, Heilbron, 1942), while in algae which have been kept in a dry condition the latter pigment is replaced by zeaxanthin (Heilbron & Phipers, 1935). A detailed examination of the pigments of the male exudate of the dioecious brown algae *Ascophyllum nodosum* Le Jol, *Fucus serratus* Linn and *F. vesiculosus* Linn, has revealed that the predominating orange pigment, unaffected by alkaline hydrolysis, is the hydrocarbon β carotene, already known to be the typical epiphasic carotenoid of the brown algae. A number of other carotenoid pigments is certainly present in very minute quantities, some of these may be isomeric forms of carotene (Carter & Gillam, 1939, Zechmeister, 1944), but although considerable amounts of algae have been employed (the exudate from 250 kg of *Ascophyllum nodosum* was extracted) it has not been possible to isolate any other pigment in sufficient quantity even for spectrographic analysis. It is of interest to note that, among the fungi, the small motile male gamete of some species of *Allomyces* (e.g. *A. javanicus*) is known to possess a conspicuous orange globule, whilst the larger female gametes are colourless. Emerson & Fox

(1940) have found that this orange colour is due to carotenoid pigments, in particular to γ carotene along with traces of isomers. Also the thick-walled sporangia produced during the sporophytic phase in the life cycle of this fungus contain a brown pigment of the melanin group and no trace of carotenoids.

In direct contrast to the results obtained with the male exudates our examination of the pigments of the corresponding female exudates indicated that chlorophyll and fucoxanthin, both typical pigments of the brown algae, are almost entirely responsible for the colour of the female gametes. Only traces of β carotene were detected in the extracts, but the fact that the egg possesses a representative sample of the pigments of the parent plant, even in differing proportions, is not unexpected. The crude concentrated extracts of both male and female gametes were largely contaminated with oily impurities, presumably terpenoid in nature, in which the carotenoids are dissolved in the cell chromatophores. (Such materials have been shown (Haas & Hill, 1933) to be present in many members of the Phaeophyceae, and to be more abundant in species growing high in the littoral zone.) These contaminants, particularly in the case of the male exudate, were undoubtedly responsible for the poor yields of β carotene obtained in a crystalline form, since chromatographic adsorption failed to separate the pigment from these oily impurities. The quantity of fucoxanthin in the female exudate was considerably less than that of the β carotene in the male, so small was the amount of the former that only from *Ascophyllum nodosum* could any solid material be obtained.

Resolution of the exudate pigments of the monoecious species *Fucus spiralis* Linn, gave results similar to those of the dioecious members examined, in that β carotene, fucoxanthin and chlorophyll were all present in the mixed exudate, the carotene presumably arising from the orange sperms and the two latter pigments from the greenish eggs.

EXPERIMENTAL

All melting points are uncorrected. The light petroleum used in these experiments had b.p. range 40–60°. Anhydrous Na_2SO_4 was employed for drying solutions, and all operations were carried out under N_2 , evaporations being effected under reduced pressure. Alumina refers to Brockmann's 'special for adsorption', MgO was always diluted with an equal bulk of Hyflo Super Cel (Strain, 1934). $\text{Ca}(\text{OH})_2$ was activated before use by heating at 130° for 1 hr. Elutions were effected with a 5% solution of methanol in light petroleum except where otherwise stated.

Fucus vesiculosus, male. A concentrate of sperms from 13 kg of the whole fruiting alga was obtained by allowing the alga to stand for 12–24 hr in a moist atmosphere, and then washing off the exuded material by dipping into a small volume of water. This treatment of the fruiting plants is reasonably comparable with the intertidal conditions on the shore, when the expulsion of the repro-

ductive bodies from the conceptacles is known to take place. The concentrate was centrifuged to remove as much water and mucilage as possible, and the orange residue then ground with anhydrous Na_2SO_4 . Treatment of the moist cake so obtained first with methanol and then with light petroleum gave a solution which contained almost all the pigments of the original exudate. The petroleum-methanol solution was washed with water, the resulting light petroleum solution* was then shaken with an equal volume of 95% methanol, but no hypophase pigments could be separated. The washed and dried light petroleum solution was adsorbed on a column of calcium hydroxide, the chromatogram being developed with the same solvent. The final chromatogram was essentially similar to those obtained from the exudates from male *Ascophyllum nodosum* and *Fucus serratus* (see later), consisting of a lower orange carotene zone in preponderating amount and a complex series of narrow bands in the upper portion of the column. The lower zone was eluted, and, after washing, the solvent was removed. Crystallization of the red oily residue yielded carotene (9 mg), which on recrystallization gave crystals of impure β carotene (7 mg), absorption maxima in carbon disulphide 517, 484 and 454 m μ .

Ascophyllum nodosum, male. The suspension of sperms and antheridia from some 250 kg of the fruiting alga was treated by a technique similar to that described under *Fucus vesiculosus*, male, except that the suspension was filtered through coarse fluted filter papers which were then extracted with acetone-light petroleum (1:4).

By elution of the carotene zone of the chromatogram and crystallization of the resultant red oil (140 mg) from benzene-methanol, β carotene (16 mg, m.p. 178°) was obtained as red leaflets with a greenish metallic lustre, absorption maxima in carbon disulphide 517, 487 and 455 m μ . (Carter *et al.* 1939)

Traces of fucoxanthin and chlorophyll, insufficient for characterization except by their behaviour on a chromatogram, were found in the hypophase.

Fucus serratus, male. The exudate was removed from the apices (5 kg) by wiping with 'Kleenex' cellulose tissues, which were subsequently extracted with light petroleum, into which solvent the entire pigment was transferred.

Treatment of this solution by the technique already described gave a red oil (150 mg), which on crystallization yielded β carotene (3 mg), m.p. 172°. Absorption maxima in light petroleum 479–80 and 449 m μ , in carbon disulphide 516 and 484–5 m μ .

The mother liquors obtained after separation of the crude carotene still contained much pigment, which was largely contaminated with oily impurities. In spite of saponification and several reabsorptions this material failed to yield any further quantity of crystalline product.

Fucus vesiculosus, female. The exudate from 8 kg of fresh fruiting alga was treated by the technique developed with male *F. vesiculosus*, with the exception that the Na_2SO_4 cake was extracted first with acetone and then with ether. The combined extracts were washed, the dried solution was evaporated and the residue adsorbed, from

benzene solution, on a column of alumina, the chromatogram being developed with benzene. A pale yellow filtrate passed through the column, this consisting of a trace of β carotene, which in light petroleum solution exhibited absorption maxima at 477 and 446 m μ . The only pigments adsorbed on the alumina were fucoxanthin and chlorophyll. The reddish brown zone of the former, occurring between two greenish bands, gave on elution the characteristic fucoxanthin blue coloration with HCl. Absorption maxima in carbon disulphide were seen at 506–7, 477 and 446 m μ . A mixed chromatogram with pure fucoxanthin on alumina gave only one zone on development.

Ascophyllum nodosum, female. A concentrate of the exudate from 250 kg of the fresh fruiting alga was treated in a similar manner to that described for *Fucus vesiculosus*, female, except that the residue obtained after centrifuging was extracted directly and not treated with Na_2SO_4 .

A trace of β carotene was obtained as the epiphase pigment, while the hypophase yielded chlorophyll and impure fucoxanthin (5.5 mg). This pigment exhibited the characteristic fucoxanthin coloration on treatment in ether with 2.5N HCl. Absorption maxima in carbon disulphide occurred at 509, 478 and 453 m μ . (510, 477 and 455 m μ given by Heilbron & Phipers, 1935)

Fucus serratus, female. The exudate of the sexually active apices (4.5 kg) was extracted in a manner similar to that described for *F. serratus*, male. No pigment could be extracted from the cellulose tissues with light petroleum, but extraction with ethanol gave a dark green solution.

A trace of an epiphase pigment, probably carotene, was present, whilst the main pigments, obtained in the hypophase fraction, were chlorophyll and fucoxanthin. The latter was largely freed from chlorophyll by chromatography, and the pigment characterized by the blue coloration with HCl. Absorption maxima in carbon disulphide occurred at 508 and 473 m μ , with one at 670 m μ due to presence of a little chlorophyll.

Fucus spiralis, male and female. A concentrate of the exudate from 18 kg of the fresh, sexually active alga was obtained and treated as described under *F. vesiculosus*, male. The Na_2SO_4 cake was extracted first with acetone and then with ether, the combined extracts were washed, dried and evaporated and the residue was partitioned between equal volumes of light petroleum and 95% methanol. The dark green petroleum phase was washed with 95% methanol, then with water and dried, and the concentrated solution adsorbed on a column of Ca(OH)_2 , the chromatogram being developed with light petroleum. The chromatogram consisted of three uppermost greenish brown zones, then a series of very small bands, while the lower part, containing most of the carotenoid pigment, consisted of a single orange zone of β carotene. Elution yielded insufficient material for crystallization, but the light-petroleum solution showed absorption maxima at 477 and 447 m μ . (see footnote in previous column). The methanol hypophase was diluted with water and extracted with ether, the ethereal solution was washed, dried and evaporated, and the residue transferred to a small volume of dry benzene. This latter solution was adsorbed on a column of Ca(OH)_2 , and the chromatogram developed first with benzene and then with ether-benzene (1:4). The fucoxanthin formed an orange zone below the greenish zones, and on elution it gave the blue coloration with HCl. Absorption maxima in carbon disulphide 476 and 446–7 m μ .

* This solution, and light petroleum solutions containing β carotene from the other algae, before crystallization, exhibited absorption maxima at 477 and 447 m μ , while similar solutions in light petroleum (b.p. 60–80°) showed maxima at 480 and 448 m μ . (cf. Carter & Gillam, 1939)

DISCUSSION

The pigmentation of the respective gametes of the various *Fucus* examined is remarkably uniform. This result is not unexpected since the pigments and sterols of the whole plants of the order are similarly uniform (Carter *et al.* 1939). However, the preponderance of β carotene in the sperm is unusual enough to promote speculation as to the function of that pigment.

In the *Fucus*, the sexual reproductive phase occurs at a particular period of the year extending in some species over several months, and in this respect the annual fruiting period is believed to spread over a longer period than in most other algae. The reproductive phase is usually followed by a period of vigorous vegetative growth, but elongation does not occur to any perceptible extent while the reproductive organs are being produced. On their discharge into sea water the sperms are extremely active and motile. The period of activity of the sperms, however, begins within the antheridial walls before final release through the apical pore of the antheridium. It might be expected that the energy expended by the sperm is obtained entirely, or in part, from light energy absorbed by the β carotene which it contains, but evidence from some preliminary experiments which have been carried out suggests that the light absorbed by the β carotene of the sperms after discharge has no connexion with the ciliary movements or with the fertilization process itself.

These experiments on the motility of *Fucus* sperms, using light of various wave lengths, suggest that the nature of the illumination affects activity only in so far as the velocity of movement and the length of the period of motility are concerned. There is evidence that the sperm suspension retains its vitality and fertilizing power for a relatively longer period if the activity is lessened, and loses it rapidly if the movement is violent. It seems, therefore, that the sperm is fully equipped with a store of energy at the time of discharge and its capacity for movement is determined by its activity and the rate of utilization of this energy store. This is in agreement with experiments on animal sperm activity, since

Cohn (1918) showed that the total carbon dioxide production of animal spermatozoa is the same whether their life be long or short.

The accumulation of carotenoid pigments in the reproductive phase and sexual organs is not unusual, and Murneek (1934) has demonstrated that the concentration of carotenoids reaches a maximum in the leaves of *Cosmos*, *Salvia* and *Soja* at about the time of flowering, after which there is a decrease. He points out a suggestive analogy with animals in which carotenoids are present in various organs and secretions usually associated with reproduction and in which the yellow pigments pass from other parts of the body to the reproductive organs, e.g. poultry and cattle.

Different proportions of pigments are reported in some male and female plants, and it appears (Murneek, 1934) that more yellow pigments are present in the female than the male of certain dioecious flowering plants (*Rhamnus*) and fungi (*Mucor*), whilst Delano & Dick (1937) maintain that for some dioecious plants (willow and white poplar) there is more carotene and chlorophyll in the leaves of the male plant than in the female.

The physiological significance of carotenoid pigments in the plant is far from being understood, but within recent years a most important function of these pigments has been revealed. Picrocrocin, crocin and *cis-trans* crocetin dimethyl esters have been demonstrated to play a fundamental role both in the copulation of the male and female gametes and in the subsequent sex determination in *Chlamydomonas eugametos* (Kuhn, Moewus & Jerchel, 1938, Kuhn, Moewus & Wendt, 1939, Moewus, 1938).

SUMMARY

The predominating colouring matter of the male gametes of various brown algae has been shown to be β carotene, fucoxanthin and chlorophyll; are the pigments of the female gametes.

We are indebted to the Rockefeller Foundation for a grant in aid of this research. We wish to acknowledge the assistance given by the late Dr C. G. Elot in the examination of *Fucus serratus*, female, and by Dr H. M. David in the laborious task of collecting material for this investigation.

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Quantitative Micro-analysis of Amino-acid Mixtures on Paper Partition Chromatograms

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The successful separation of amino acids on paper partition chromatograms (Consden, Gordon & Martin, 1944) naturally led to attempts to employ similar methods quantitatively. The methods described below are presented in a somewhat unfinished state, as external circumstances compelled an interruption of the work. In particular, no attempt has been made so far to devise an analytical method suitable for a normal protein. Only the simpler problem has been attempted of analyzing quantitatively such relatively simple mixtures of amino acids as can be separated using one dimensional paper chromatograms. No doubt the method could be applied to more complicated mixtures, but preliminary separations, by paper chromatography or other methods, would be required.

PRELIMINARY EXPERIMENTS

Several methods adapted to micro estimations were investigated. Attempts were made to utilize the colour reaction with ninhydrin upon paper. It was found possible to wash off the colour from the paper with a variety of solvents, but the estimation of colour in a photoelectric microcolorimeter did not give consistent results. These experiments, however, showed that the coloured product consisted of several components whose proportions depended upon the exact conditions under which the colour was developed.

Attempts were next made to utilize the Folin reaction, both the original Folin (1922) technique and the modification of Frame, Russell & Wilhelm (1943), again using a photoelectric microcolorimeter. Both methods were reasonably satisfactory for such quantities of pure amino acids as are available on a paper chromatogram, but when amino acids were washed from the chromatogram, interfering substances, apparently derived both from the paper and the solvents, rendered the results grossly inaccurate.

Nitrogen estimation by the Kjeldahl method was also tried. This method is very attractive theoretically, as it was hoped that after a qualitative analysis the chromatogram could be cut up and a quantitative estimate obtained from the amount of nitrogen in each coloured spot. The

technique used was that of Hawes & Stavinsky (1942). The scale of working was reduced by a factor of 10, and satisfactory results were obtained, if the starting material was $(\text{NH}_4)_2\text{SO}_4$ and the incineration omitted. However, the atmosphere of the laboratory proved to be so heavily contaminated with the nitrogenous solvents used in the chromatography that satisfactory incinerations could not be performed. It was not even possible to determine whether the nitrogen blank of the paper was low enough to render the method feasible.

Titration in glacial acetic acid (Harris, 1935) was next employed. The amino acid was washed from the paper into small titration tubes and, after evaporating in a vacuum desiccator and dissolving in 20 μl glacial acetic acid, it was titrated with 0.01N HClO_4 in acetic acid, using crystal violet as indicator (Nadeau & Branchen, 1935). Excellent results were obtained with pure amino-acids, quantities of 15–30 μg being titratable with an error of 1%. The paper, however, contained such large amounts of acids and bases, which could not be removed by preliminary washing, that the method could not be used on the material washed from the paper.

To avoid interference with contaminating substances, the relatively specific complex formation of the amino acids with copper was then tried. The technique used was essentially that of Pope & Stevens (1939), which is a modification of the method of Kober (1912). The amino acid was added to a suspension of copper phosphate in a phosphate borate mixture, causing the copper to go into solution as the amino acid copper complex. After filtration, the copper in solution was estimated iodometrically. Stoichiometric results were obtained for almost all the amino acids, with a ratio of two amino acid molecules to one atom of copper. It was not found possible to reduce the scale of the iodometric titration to the micro quantities available, though good values on 100–200 μg of pure amino acid were obtainable. However, even in this case, reducing material derived from the paper interfered with the iodometric titration and prevented satisfactory estimation of the material derived from the paper chromatograms.

Finally, the polarograph (see Kolthoff & Lingane, 1941) was used to estimate the copper complex, in place of the iodometric titration. This proved to be sufficiently specific to give useful though not highly accurate results. As has already been said, the method has not been fully worked out and it is hoped that further experience will increase the accuracy and the availability of the method for complex mixtures.

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EXPERIMENTAL

Polarographic technique

In the initial stages a simple galvanometer, fitted for visual observation, was used with the dropping mercury cathode. Later, a Cambridge Instrument Company's photographic instrument was used. This is convenient when complete current voltage curves are required, but for routine use, as described below, the simple galvanometer is as satisfactory, since observations are made at one potential only.

The dropping mercury cathode used throughout this investigation had the following characteristics: Height of mercury 62 cm, drop time 2.4 sec, rate of flow 2.70 mg/sec in N KCl on open circuit. No attempt has been made to work at a constant temperature, but any given set of measurements has been completed within so short a time that temperature variations have been small. There is, however, an obvious advantage to be gained by working at a constant temperature. The polarograph cells were of two sizes, one 9 mm internal diameter, 8 cm long, and the other 3 mm internal diameter and 3 cm long, each was fitted with a platinum anode sealed into the bottom. The larger cells required 1–2 ml and the small ones, which have to be completely filled with solution before the electrode is introduced (to avoid air bubbles), required 0.2 ml.

Throughout, sulphite has been used to remove O_2 , and has proved more convenient than H_2 or other gas, particularly with the smaller volume of solution. Since, with our solutions, the step at about 0.0 V before the Cu step begins has been too steep to permit accurate measurement, the zero of the instrument, with the dropping cathode disconnected, has been employed as the reference point.

Supporting electrolyte

A solution of 50 g $CuCl_2 \cdot 2H_2O$ was added with stirring to 200 g $Na_2HPO_4 \cdot 12H_2O$ in about 1.5 l of distilled water, and NaOH was added to pH 9.0. The precipitate was filtered on a large Buchner funnel, washed on the filter with 2% borax solution, and then made up to 1 l with 2% borax solution. This stock suspension can be kept indefinitely. 5 ml of this suspension, with 1.70 g $Na_2HPO_4 \cdot 2H_2O$ and 2 ml of the starch solution of Pope & Stevens (1939) were made up to 200 ml with 2% borax. This suspension, which should be made up weekly owing to growth of moulds, forms the supporting electrolyte for the polarographic measurements. The amino acid was added to a known volume of this suspension and left for 30 min. or more (it can be left for 24 hr without further change), 1 or 2 ml. were now added to the polarograph cell and a small knife point (c 2 mg) of Na_2SO_3 was dissolved by stirring with a glass rod (1 mm diam with a bent end) which was rolled between fingers and thumb. The dropping electrode was now put into position and allowed to drop for 2 min with no current passing. The current at -0.5 V was then observed at 1 min. intervals until steady for 1 min., and then recorded on the photographic drum. The instrumental zero, the dropping electrode having been disconnected, was then immediately recorded on the same segment of the drum. With the large cells the observation was discarded if the current had not become steady within 15 min, and with the small tubes within 5 min. Usually

the current had become steady 3 min after the electrode had been put in.

The shape and height of the current voltage curve depends upon the composition of the supporting electrolyte. In the absence of starch, two pronounced maxima are shown at about -0.3 and -1.3 V. With about 0.01% of starch, both maxima are suppressed, but the height of the step is not much affected (Fig 1). With higher concentrations of starch the height of the step is greatly reduced and the shape of the curve changed. With the solution used there is a long straight portion from 0.3 to -1.2 V. Measurements have been made at -0.5 V, which has been arbitrarily chosen within this range.

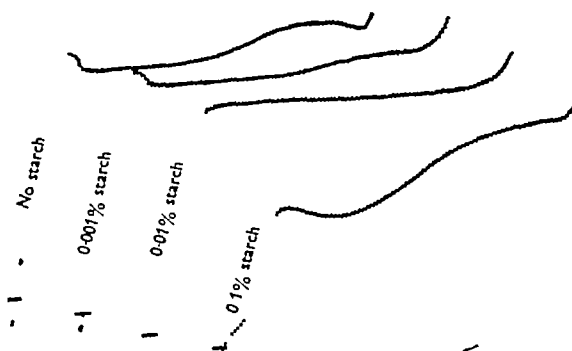


Fig 1 Effect of starch on the current-voltage curve of the glutamic acid copper complex. The short cross line near the origin of each curve is the 'instrumental zero'.

The amount of Na_2HPO_4 in the supporting electrolyte affects the height of the step. In its absence, copper phosphate is sufficiently soluble to give a large blank of ionic copper. In the presence of high concentrations of Na_2HPO_4 , the amount of the ionic copper is so small that an appreciable proportion of the amino acid does not react, and low results are obtained both polarographically and by iodometric titration. This is true also of a CuO suspension in 2% borax.

The original Pope & Stevens (1939) mixture gave satisfactory results in the polarograph if the solution had been filtered from copper phosphate. If the suspension was used without filtering, at about -0.5 V the current began to increase, and at higher voltages became very large and independent of the amount of copper in solution. The value of the current in the supporting electrolyte in the absence of amino acids is very variable. The presence of as little as $2.5 \times 10^{-5} M$ amino acid greatly reduces the variability. For this reason, no blanks are included in the comparisons given. Perhaps because of the variability it has not been possible to demonstrate a consistent curvature of the current/concentration curve at the lower concentrations of amino acids. When an amino acid on a paper chromatogram was to be estimated, the paper containing the amino acid, which had an area of about 40 cm², was folded, cut into pieces of 10 mm² and introduced into a centrifuge tube by means of a wide necked funnel, 3 ml of the supporting electrolyte were then added, which just covered the paper. It was then allowed to stand overnight to ensure that diffusion of the amino acid from the paper to the copper

phosphate was complete. The paper and the copper phosphate were centrifuged down and the supernatant liquid was transferred to the polarograph cell and the measurement made as before.

Chromatographic technique

The method described by Consden *et al* (1944) and Consden, Gordon & Martin (1947) has been followed with out essential change. Whatman no 4 paper has, however, been used and HCN has been employed to eliminate the 'pink fronts', where necessary. Wide strips were used, so that the amino acids would not approach the edge of the paper where irregularity of running is likely to occur.

A sheet of filter paper was marked out with pencil into, say, ten strips, each 4 cm wide. The solution to be analyzed was applied with a microburette to strips 3, 5, 7 and 9, about 7 cm from one end. The amount, the same for each strip, should be enough to contain about 20 μg of each amino acid to be estimated. To strips 2, 4, 6 and 8 was applied the control mixture, with the amounts on each strip in arithmetical progression, the mean amount being similar to that believed to have been put on strips 3, 5, 7 and 9. Strips 1 and 10 had amounts of control mixture equal to that on strip 8. After development of the chromatogram, and very thorough drying, strips 1 and 10 were cut off, treated with ninhydrin and used as guides for cutting up the other strips. The guide strips have more amino acid on them than most of the other strips, and hence the separation is probably better on the other strips.

Since there was no direct indication of the proper place to cut, merely an inference from another chromatogram, the line of cutting carried an appreciable error. In order to ensure that the spots are well separated, the time of running of the chromatogram was increased as compared with that employed in qualitative work. If the guide strips show any considerable difference between each other or if the spots are not well separated, that experiment should be abandoned.

In all the measurements a direct comparison has been made between the material under analysis and a control mixture which simulates it as closely as possible. By such a comparison, rather than by attempting an absolute measure, various errors, e.g. those due to variation of temperature or to adsorption by the paper, were minimized.

After the polarographic measurements had been completed in the way described above, the current at 0.5 V was plotted against the amount of amino acid for the control series. The best straight line was then drawn (by inspection) through these points (Figs 8 and 9). This procedure is justifiable over the range of concentration employed, since no consistent curvature has been discovered. The mean of the current readings of the mixture under analysis was then plotted on this line and hence the amount of amino acid present was inferred.

RESULTS

The results of application of these methods are presented graphically below. Figs 2 and 3 show the relation between current and concentration of amino acid, the solution being added directly to the copper phosphate suspension and used without

filtration after addition of solid sulphite. No explanation has been found of the variation of both zero current and slope from one experiment to the

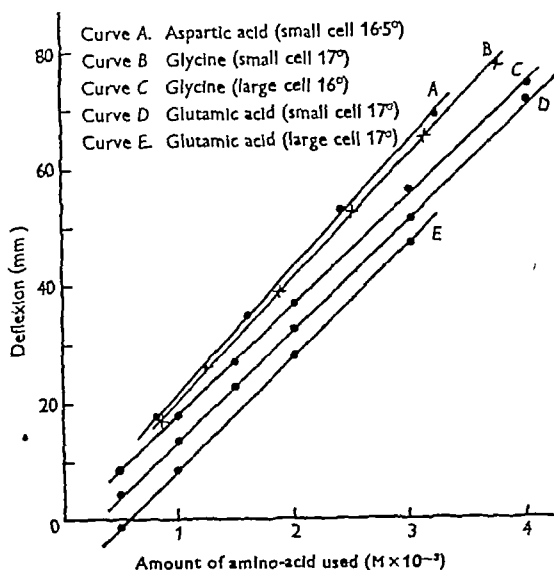


Fig 2 Relation between current and concentration of amino acid at -0.5 V using large and small cells. The zero of the glutamic acid in small cells has been reduced by 5 mm., and glutamic acid in large cells by 10 mm., to avoid overlapping. Sensitivity of galvanometer $1/50$.

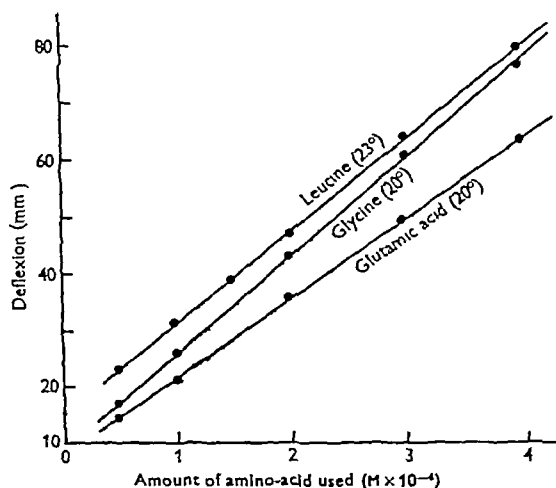


Fig 3 Relation between current and concentration of amino acid. The zero of the leucine line has been raised 5 mm. to avoid overlapping. Sensitivity of galvanometer $1/7$.

next, while the results within a given experiment (represented by a single line on the graph) are much more consistent. The copper complex of leucine is too insoluble to be used satisfactorily alone at 10^{-3} M

In Fig 4 a comparison is made between measured amounts of glycine put on a chromatogram, alone and mixed with glutamic acid. The chromatogram was run for 41 hr in phenol coal gas 0.3% NH_3 . The extra manipulation and lower concentration has resulted in a decreased consistency of the measurements. Large cells were used here and throughout the remainder of the work.

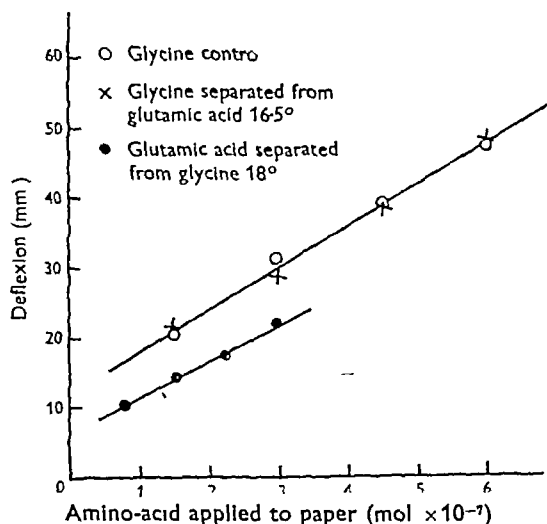


Fig 4 Glycine and glutamic acid separated on chromatogram Galvanometer sensitivity 1/5

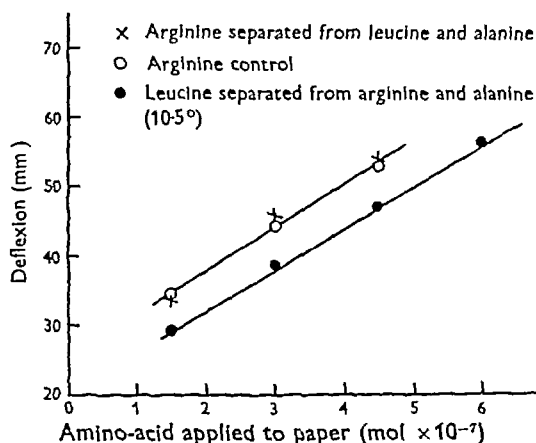


Fig 5 Arginine, leucine and alanine separated on chromatogram Galvanometer sensitivity 1/3

In Fig 5 a comparison is made between measured amounts of arginine put on a chromatogram alone and mixed with leucine and alanine. Alanine results were lost. Chromatograms were run 41 hr in *s*-collidine diethylamine HCN.

In Fig 6 a comparison is made between measured amounts of proline put on a chromatogram alone and as a mixture of equimolar amounts of proline, valine, leucine, glutamic acid, aspartic acid, phenylalanine, tyrosine and ornithine. The chromatogram

was run for 67 hr in *s*-collidine diethylamine HCN.

In Fig 7 a comparison is made between glutamic acid alone and aspartic acid alone and both, in the same mixture as used in the experiment described

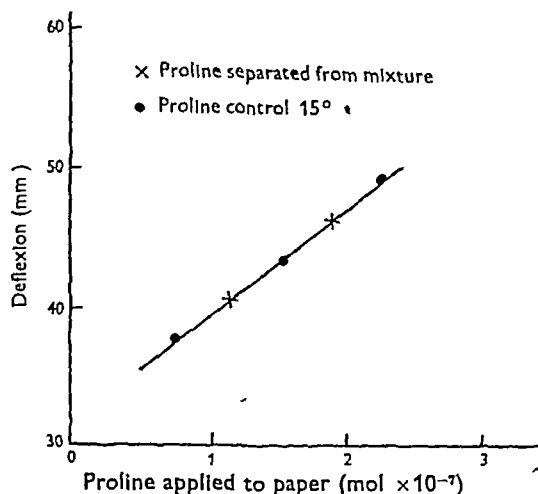


Fig 6 Proline alone and separated from a mixture Galvanometer sensitivity 1/3

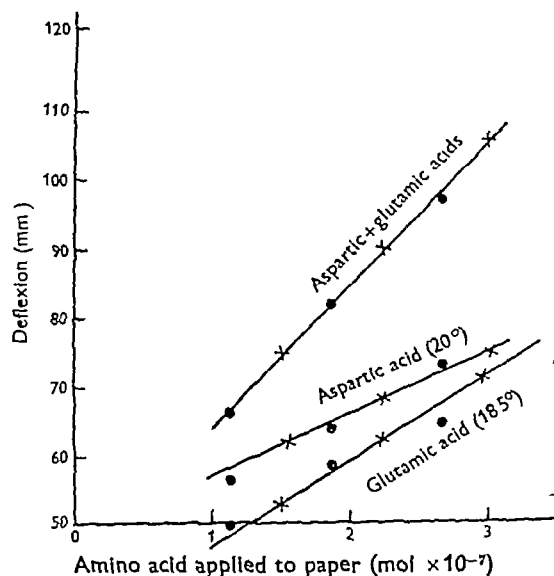


Fig 7 Glutamic and aspartic acids separated from a mixture of amino acids Galvanometer sensitivity 1/2 \times , controls of the single amino acids, \bullet , amino acid applied as mixture

in Fig 6. The chromatogram was developed for 67 hr with phenol-0.3% NH_3 . The aspartic and glutamic acids were not, on this occasion, perfectly separated from each other as judged from the guide strips, though well separated from the six other amino acids. For this reason the sum of the deflections for aspartic and glutamic acids has also been plotted for the mixture and the controls.

Analysis of gramicidin S

Figs 8 and 9 show an analysis of gramicidin *S* Syngé (1945) concluded that this substance consists of equimolecular amounts of ornithine, proline, valine, leucine and phenylalanine, perhaps with 1 or 2 mols of water

10.8 mg of air dry gramicidin *S* were weighed into a small tube with 1 ml. of 8*N* HCl. The tube was sealed and put in an oven at 105–110° for 48 hr. After opening, it was dried in a vacuum desiccator over KOH. To remove further

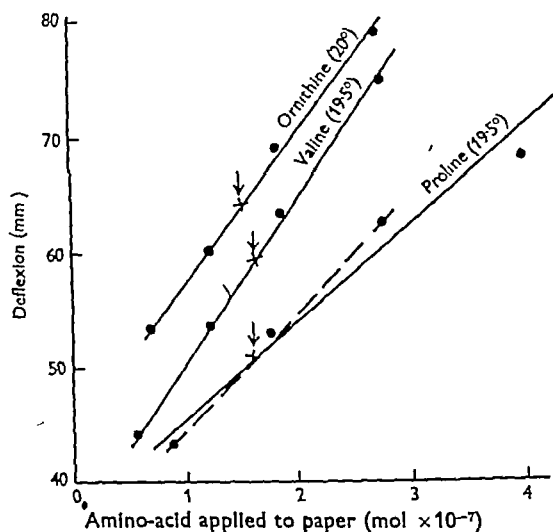


Fig 8 Analysis of gramicidin *S* x, mean of five determinations on 10 μ l of hydrolysate, other points single values of control mixture Sensitivity of galvanometer 2/3

HCl, water was added twice, and evaporated in the desiccator. Finally, 1.013 g of water were added 10 μ l of the resulting solution were used on each chromatogram spot. Since altogether nine spots were used, 0.96 mg of gramicidin *S* was actually consumed in the analysis.

The control mixture contained 12.8 mg DL-proline, 9.0 mg DL-valine, 12.7 mg L-ornithine hydrochloride, 13.3 mg DL-phenylalanine, 9.8 mg DL-leucine made up to 10 ml. The ornithine was a rather dark coloured specimen prepared from arginine but free from other amino acids. The other amino acids were supplied by Roche Products Ltd and believed to be pure.

Fig 8 shows the results obtained with *s*-collidine-diethylamine HCN as solvent. The chromatogram was run for 67 hr. Comparison was made between the synthetic mixture, run at four different levels, and five lots of gramicidin *S* at the same level of 10 μ l of solution. The deflexions given for the gramicidin *S* were ornithine, 53.5, 54.0, 54.5, 54.5, 54.5, mean 54.2 mm, proline, 50.0, 51.5, 51.1, 52.0, 50.0, mean 50.9 mm, valine, 59.5, 59.0, 59.8, 59.0, mean 59.3 mm (one lost through cracking of centri-

fuge tube). The mean values are plotted on the lines given by the control mixture.

Fig 9 shows results obtained with benzyl alcohol-HCN. The chromatogram was run for 41 hr. Comparison was made between five levels of control mixture and four spots of 10 μ l of gramicidin solution. The deflexions given for the gramicidin *S* were leucine, 39.0, 39.0, 39.5, 38.5, mean 39.0 mm, phenylalanine, 36.0, 35.5, 35.0, 35.6, mean 35.5 mm. The mean values of the gramicidin *S* deflexions were plotted on the lines given by the control mixture.

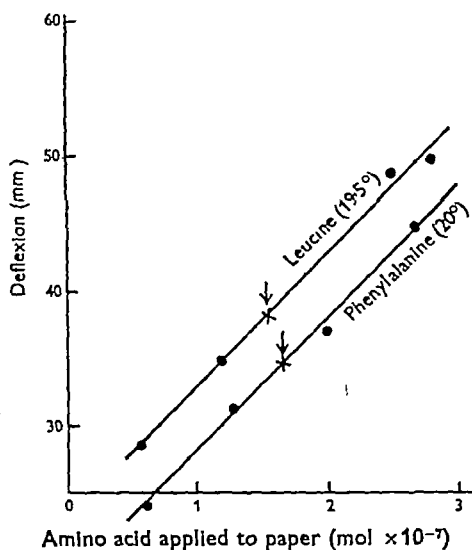


Fig 9 Analysis of gramicidin *S* x, mean of four determinations on 10 μ l of hydrolysate, other points single values of control mixture Sensitivity of galvanometer 2/3

From Syngé's formula, the mols of each amino-acid contained in 10 μ l of the hydrolysate are 1.69×10^{-7} assuming 1 mol H_2O , and 1.64×10^{-7} assuming 2 mols H_2O . The amounts of amino acids determined from Figs 8 and 9, in terms of mols $\times 10^{-7}$, are: Found proline, 1.62, valine, 1.65, ornithine, 1.55, leucine, 1.53, phenylalanine, 1.67. Expected for all amino acids 1.64 mols.

DISCUSSION

An advantage of the use of the lower concentrations of amino acids which are described here, compared with those used by Kober (1912) or Pope & Stevens (1939), is that amino acids forming relatively insoluble copper complexes, e.g. leucine and phenylalanine, can be used without the addition of a solubilizer such as glycine or glutamic acid, the use of which is, because of the large amount which has to be added, attended with a large increase of error. Colorimetric methods of measuring copper are available which could almost certainly be made to work at the micro level required, but the labour involved in these is great compared to the use of the

polarograph directly on the suspension. Apart from its use for amino acids, the method should be applicable to peptides, provided authentic specimens of these exist for comparison. For the amino acids, the value of the method lies principally in the small amount of material required for analysis, though at present the whole range of amino acids have not been dealt with simultaneously. For certain amino acids the microbiological methods, while using comparable amounts of material, are more accurate. For peptides it is improbable that it will be worth while developing the microbiological methods.

The results obtained in the analysis of gramicidin *S*, though intended primarily as a test of the method of analysis, may be considered to confirm the formula proposed by Syngé (1945). The material consumed in the analysis here (0.96 mg) is about 10% of that which Syngé employed, using partition chromatography of acetamido acids on silica columns.

SUMMARY

1 Various methods have been investigated in an attempt to render quantitative amino acid analysis by partition chromatography on paper.

2 A polarographic modification of Kober's method for the estimation of amino acids as their copper complexes is described. A few micrograms only are required.

3 Estimations of several amino acids in a number of simple mixtures are presented. An analysis of gramicidin *S* using 1 mg only, confirms the formula proposed by Syngé.

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The Amino-acid Composition and Titration Curve of Collagen

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The amino-acid composition of gelatin has been extensively studied, but until recently little attention has been paid to that of its precursor collagen, the natural protein of skin. Some of the analyses of collagen which have been reported were carried out by methods which are now considered unsatisfactory, and in no case have most of the major component amino-acids been determined in one sample of the protein. Further, most of the analyses have been made on collagens which had been given an alkaline or enzyme treatment, or both, to remove the keratins, elastic fibres and reticular tissue which occur in close association with it, and there is a risk

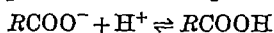
that such treatments will cause some modification of the collagen.

For the present study, a sample of collagen has been prepared with the minimum of chemical treatment, since it was considered that the presence of small amounts of elastic fibres and reticular tissue would lead to less error than the treatments necessary to bring about their removal. Also, it seems doubtful whether it is possible to remove these proteins completely by any of the methods which have been suggested. On this preparation Chibnall and his collaborators (Chibnall, 1946; Macpherson, 1946; Rees, 1946; Tristram, 1946) have determined

the basic and hydroxyamino acids, and the following neutral amino acids alanine, leucine, isoleucine, valine, phenylalanine and proline, the present authors have determined total N, amino N, amide N, glutamic and aspartic acids, and methionine

The acid and basic amino acid contents may also be deduced from the titration curve of a protein. This procedure has an advantage over analysis for the individual amino acids in that it gives information regarding the reactive groups, including terminal groups, in the intact protein, and comparison of the results with those obtained by direct analysis can give information regarding the way in which the amino acids are linked in the protein molecule (Chibnall, 1942, Cannan, Kibrick & Palmer, 1941, 1942)

It must be observed, however, that the titration curve of a fibrous protein only gives an exact indication of the titratable groups when it is determined in the presence of salts (Speakman & Hirst, 1933, Jordan Lloyd & Bidder, 1934, Steinhardt & Harris, 1940). This effect of salt is due in the first place to its influence on the Donnan equilibrium. The effective pH value for the equilibrium



is the pH value in the protein phase, in the absence of salts this pH value is higher than that in the external solution, except at very low pH values, and so the binding of hydrogen ions is less than that corresponding to the external pH value. It has also been suggested (Gilbert & Rideal, 1944) that stoichiometric binding of hydrogen ions by fibrous proteins only occurs when some anions are also bound in order to lessen the large potential which would otherwise exist between fibre and solution. The addition of salts, by increasing the concentration of anions, will facilitate this binding, and thus favour stoichiometric binding of hydrogen ions (see also Steinhardt & Harris, 1940). Similar arguments apply to titration with alkali. The concentration of salt necessary for stoichiometric binding depends on the protein, with collagen it has been shown to be 0.1 M or greater (Retterova, private communication).

In the present investigation the titration curve has been determined in the absence of salts, and also in the presence of 0.5 M sodium chloride.

METHODS

Preparation of collagen The back area of a freshly flayed ox hide was cut into pieces (c. 18 × 12 in.), which were washed in a revolving glass drum, first with water and then with 10% NaCl for 30 min. The pieces were left stationary overnight in a fresh NaCl solution, drummed for 30 min. in this solution, washed in several changes of distilled water, and dehydrated with acetone. The grain layer (containing the hair roots, and the greater part of the muscle and elastic fibres) and a thin layer from the flesh side were split off,

and the remaining material cut into 1 cm. cubes. The cubes were degreased with three changes of light petroleum (b.p. 40–60°) at room temperature for 6 days, washed with successive changes of distilled water, and dehydrated with acetone. The purified collagen contained ash 0.03, moisture 23.0 and grease <0.1%.

Analyses Total N was determined by the method of Chibnall, Rees & Williams (1943), and amide N by the method of Bailey (1937) and Lugg (1938). The amino N was determined by the Van Slyke manometric procedure, using an auxiliary reaction vessel as described by Doherty & Ogg (1943). To ensure thorough wetting of the sample, the finely shaved collagen (0.15–0.20 g.) was placed in the extraction vessel with 2 ml. of water, the vessel evacuated while shaking, and left under vacuum overnight. The vessel was re-evacuated immediately before the determination.

Dicarboxylic acids were determined by the method of Consden, Gordon & Martin (1948), and methionine by the method of Baerstein (1932).

Titration curves Collagen (0.75 g.) was placed in 75 ml. of solutions containing measured volumes of HCl and NaOH in resistance glass vessels at 20°. For the titration curve in the presence of salt, sufficient NaCl was added to give a final Cl⁻ concentration of 0.5 M in the acid solutions, and a final Na⁺ concentration of 0.5 M in the alkaline solutions. After 3 days, in which time equilibrium was known to have been reached, samples were withdrawn for pH determinations, and for titration with acid or alkali using bromocresol purple as indicator. When the final solution was too dilute for titration (between pH 3 and 11) the concentration of acid or alkali was obtained from the pH by the use of blank curves for water and for 0.5 M NaCl, thus avoided the necessity of choosing suitable activity coefficients for the two systems. In the more concentrated solutions, the amount of acid or base bound by the collagen is small compared with that remaining in solution, and larger samples of collagen (2 g. in 100 ml.) were used.

pH values were determined at 20° using a glass electrode assembly mounted in a thermostat (Coates, 1945). pH values above 9.0 were determined with a glass electrode of low sodium error (Dole, 1941).

In determining the amounts of alkali bound, precautions were taken to avoid contamination with CO₂. The collagen was placed in the vessel shown in Fig. 1a, and the air displaced with CO₂ free air passed in by the tube B. The solution of alkali was then added, the tube A closed with a small rubber bung, and B by a rubber cap. Samples for titration were taken by inserting a pipette at A, carrying a similar rubber bung, and forcing the solution into this pipette by passing CO₂ free air into the vessel at B. The titration was carried out in a vessel through which a stream of CO₂ free air was continuously blown. In the determination of pH, the solution was forced up into the electrode vessel in a similar way (see Fig. 1b), or, in the case of the electrode system mounted in the thermostat, a sample was transferred by means of a pipette.

The soluble N in some of the solutions below pH 2.0 and above pH 12 was determined by micro Kjeldahl (Markham, 1942) to give an indication of the extent of decomposition, but no attempt was made to apply a correction, owing to lack of information concerning the origin of this N.

Estimate of errors involved in the determination Errors may arise from the following causes: (1) Difference in the pH value between the solution inside the collagen and the

external solution (2) Introduction of water with the collagen, thus increasing the total volume of solution (3) Binding of water by the collagen, thus decreasing the amount of water available for solution of the acid or alkali. (4) Decomposition of the collagen, or liberation of groups not originally present in the native collagen

Table 1 Soluble nitrogen derived from collagen after 3 days' contact with acid or alkali

In the absence of salt		In the presence of 0.5M-NaCl	
pH	mg N/g collagen	pH	mg N/g collagen
1.58	0.14	1.64	0.95
1.08	0.12	1.73	0.85
1.80	0.15	1.85	0.77
—	—	2.00	0.69
—	—	2.10	0.70
12.38	1.54	12.46	1.04
12.59	1.72	12.59	1.28
—	—	12.66	1.21
12.78	1.95	12.71	1.08
12.92	2.22	12.86	1.44
13.16	3.86	13.12	3.08

0.03 mmol/g, and at pH 2.0, 0.003 mmol/g. The fact that the curves show well defined maxima in the acid and alkaline ranges confirms that the net error must be small.

The soluble N present in the solutions (see Table 1) indicates that decomposition of the collagen was small even in the most acid and alkaline solutions. The chief source of this N is probably NH_2 from hydrolysis of amide groups. Under the conditions of the experiment the greater part of this NH_2 will be titrated with acid, thus compensating for the alkali bound by the freed carboxyl groups, and hence such decomposition will not affect the calculation of the amount of alkali bound by the original collagen.

RESULTS

Amino-acid composition

The collagen, prepared as described, may be considered to be a reasonably pure sample. Of the other proteins and tissues present in the fresh skin, albumins and globulins will be removed by treatment in sodium chloride solution (Kritzinger, 1948), and the greater part of the keratins, elastic fibres,

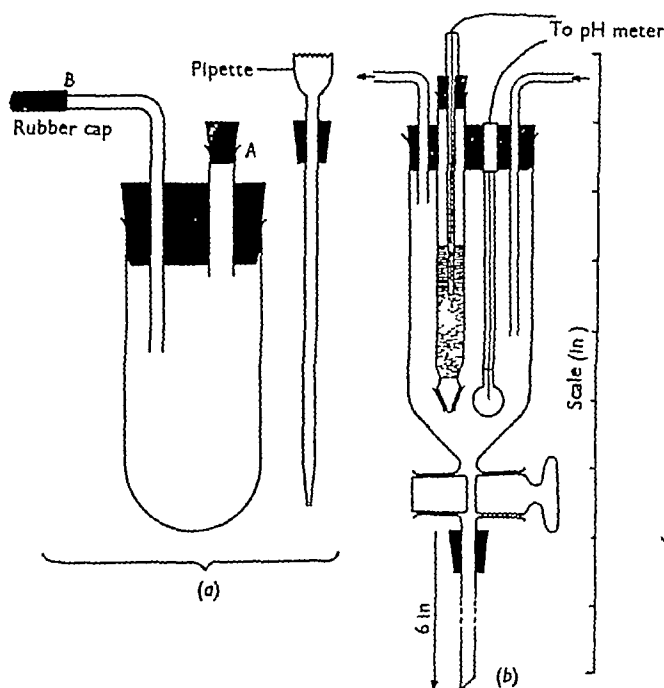


Fig. 1 Apparatus used for the determination of titration curves

The first of these errors will be appreciable in the absence of salt, and may in part be the cause of the difference in the shape of the curves obtained with and without salt. In the presence of 0.5M NaCl it is negligible (Retterova, private communication). Since the second source of error partially compensates for the third, no correction was made for it. As the volume of solution is large compared with the amount of collagen, the net error must always be small. Assuming the collagen binds 50% of water (Cheshire & Holmes, 1942), the error at pH 1 would be of the order of

muscle fibres, sweat glands, fat cells, etc. will be removed with the grain layer (Dempsey, 1946). The remaining impurities are, therefore, a small amount of reticular tissue and some elastic fibres, the presence of which should not greatly affect the results obtained.

The total N of the present collagen preparation is rather higher than the value generally quoted for skin collagen, but is the same as that obtained by

Table 2 *Analyses of collagen*

	N as % protein N	g/100 g collagen	mmol./g collagen
Total N	—	18.6	—
Amide N	3.5	0.66	0.47
Amino N	2.5	0.46	0.33
Methionine	0.4	0.8	0.05
Aspartic acid	3.6	6.3	0.47
Glutamic acid	5.8	11.3	0.77

Bergmann & Stein (1939) for ox hide tendon prepared in a similar manner. Earlier values for skin collagen have been determined on material which has had some treatment (alkaline or enzymic) likely to lead to loss of nitrogen from amide or guanidino

The values for the dicarboxylic acids are considerably higher than those obtained by Dakin (1920) for gelatin, and Schneider (1940) for collagen, and are of the same order as values recently obtained for gelatin by microbiological methods (Hac & Snell, 1945, Stokes & Gunness, 1945, Lewes & Olcott, 1945, Hao, Snell & Williams, 1945, Hier, Graham, Friedes & Klein, 1945). Gale (1945), using a specific decarboxylase, found an average value of 10% glutamic acid for this and other similar samples of collagen prepared by the present authors. This value is likely to be low owing to racemization of the glutamic acid during hydrolysis (Gale, 1945).

The methionine content of the present collagen is lower than that quoted by Baernstein (1932) for

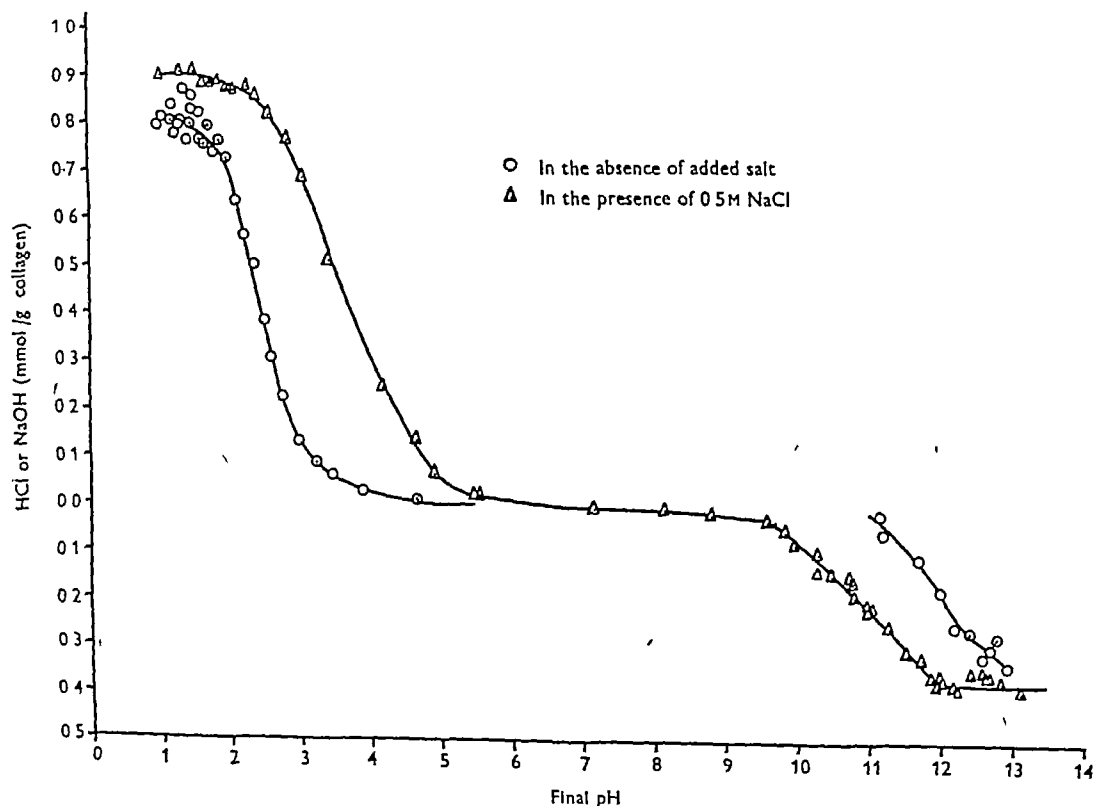


Fig 2 Titration curves of collagen with and without sodium chloride

groups. The amide N is considerably higher than values quoted for gelatin (Dakin, 1920, Chibnall, 1942), presumably because gelatin is usually made under conditions of alkali treatment which favour the breakdown of the amide groups (Highberger & Stecker, 1941, Ames, 1944). The amino N is of the same order as values obtained by Rutherford, Harris & Smith (1937) and Kanagy & Harris (1935) for hide powder.

gelatin, but is in good agreement with recent microbiological determination on gelatin (Horn, Jones & Blum (1946), 0.78%, Lyman, Moseky, Butler, Wood & Hale (1946), 0.88 and 0.83%).

Titration curves

As in previous titration curves of collagen and other fibrous proteins, the curve in the absence of salt shows a broad isoelectric range in which no

combination with acid or alkali takes place, and the acid and alkaline portions of the curve are shifted to lower and higher pH values respectively (Fig 2). In the presence of sodium chloride, however, there is a definite isoelectric point at pH 6.6–6.8, and the curve approaches that of a soluble protein. The curve shows clearly defined acid and base-binding maxima of 0.90 and 0.36 mmol/g of collagen at pH 1.5 and 12.5 respectively.

In contrast to the findings of Theis & Jacoby (1941c, 1942b), who used collagen which had been given a preliminary alkaline treatment, the acid-binding maximum is greater in the presence of salt than in its absence, and there is little indication that the two curves would meet if extended to lower pH values. There is also an indication that maximum base binding is less in the absence of salt.

Previous titration curves of collagen, all of which were determined on collagen which had received either an alkaline or enzymic treatment, show isoelectric points between 5.4 and 7.9 (Theis & Jacoby, 1940, 1941a, b, c, 1942a, b, 1943a, b, Atkin, 1937), and in those curves where definite maxima can be distinguished (Theis & Jacoby, 1941c, 1942a, 1943a, b), an acid-binding capacity of 0.85–0.90 mmol/g, and a base binding capacity of 0.38–0.45 mmol/g. The curves of alkali-treated collagen all show an appreciable base binding capacity in the pH range 6–8, which is absent in the present curve, this is also characteristic of titration curves of gelatin, and may be attributed to increase in the number of carboxyl groups resulting from hydrolysis of amide groups. The present curve can be divided into three portions, pH 1.5–4.9, 4.9–9.6, and 9.6–12.5, and from consideration of the probable pK's of the groups involved, may be interpreted as indicated in Table 3.

It is not possible to distinguish separate sections of the curve due to titration of the imidazole and α amino groups, as these are present in small amounts and do not differ greatly in pK (imidazole, pK 5.5–6.5, α amino, pK 7.5–8.5 (Cohn & Edsall, 1943)). Since collagen contains a high proportion of proline and hydroxyproline, it is possible that there are some terminal imino as well as α -amino groups, and these will titrate in a similar pH range (imino of proline, pK 9.7, imino of hydroxyproline, pK 10.1 (Cohn & Edsall, 1943)). It was also not possible to assign any portion of the curve to titration of phenolic hydroxyl groups, and in view of the small amount of tyrosine in collagen and the doubt concerning the extent to which it titrates (Fruton & Lavin, 1939, Steinhardt, 1939, Neuberger, 1943) it has been omitted from the analysis of the curve.

There is no indication that the guanidino group is titrating in the pH range covered, and the pK of this group in collagen must be greater than 14. A similar observation has been made by Lichtenstein (1940) in connexion with the titration curve of gelatin.

DISCUSSION

As far as is known to the present authors, the figures for total N, amide N and amino N are the first to be reported on skin collagen which has received no alkaline or enzymic treatment, and it is not surprising, therefore, that the values for the first two are higher than those generally quoted for collagen.

The free amino N as determined by the Van Slyke method and the number of ϵ - and α amino groups deduced from the titration curve (see Table 3) are in close agreement, but appreciably less than the value indicated by determination of the lysine and hydroxylysine in the basic amino acid fraction of

Table 3 Analysis of titration curve

Groups titrating	Method of calculation	pK from curve	Amount present (mmol/g)	
			From curve	From analysis
(a) Total basic groups	Titration from 1.5 to isoelectric point	—	0.90	0.94
(b) Imidazole	Titration from 4.9 to 9.6	7.5	0.07	0.05
Imino				—
α Amino	Titration from 9.6 to 12.5	11.0	0.34	0.33
(c) ϵ Amino*				0.39 (Van Slyke)
(d) Free carboxyl	Titration from 1.5 to 4.9	3.5	0.87	0.79
(e) α Amino + imino	(b) – 0.05 mmol imidazole groups	—	0.02	—
(f) Guanidino	(a) – (b) – (c)	>14	0.49	0.51
(g) Amide	From analysis	—	—	0.47
(h) Dicarboxylic acids	(d) + (g) – (e)†	—	1.32	1.26

* Including the side chain NH_2 group of hydroxylysine. The pK of the amino group not α to the carboxyl group in hydroxylysine is 9.50 as compared with 10.3 for the ϵ groups of lysine under the same conditions (Van Slyke, Hiller, MacFadyen, Hastings & Klemperer, 1940), and it may be assumed to titrate in approximately the same range as the ϵ amino groups of lysine.

† It is assumed that terminal carboxyl groups are equivalent to α amino + imino groups.

the hydrolysate (Macpherson, 1946) It is possible that in the intact protein some of the ϵ amino groups are bound into the polypeptide chain or for some reason not available for reaction, or alternatively the analytical figure may be too high The lysine N was determined by difference (total N of basic fraction minus arginine, histidine and hydroxy lysine N), and hence is subject to a cumulative error

oxylic acids are bound, or that there is any appreciable number of terminal amino or carboxyl groups

The analytical results obtained in this investigation, together with those previously obtained (Chubnall, 1946, Macpherson, 1946, Rees, 1946, Tristram, 1946) on the same sample of collagen, are given in Table 4.

Table 4 *Composition of collagen*

Amino acid	(1) N as % protein N	(2) As g /100 g	(3) As g residues/ 100 g	(4) As mmol./g	(5) Minimum mol. wt calc from data in (1)	(6) Assumed number of residues	(7) Apparent minimum mol wt calc from (5) and (6)
Total N	—	18.6 a	—	—	—	—	—
Amino N	2.5 a	0.46	—	0.33	—	—	—
Glycine	26.3 c	26.2	19.9	3.50	286	136	38,880
Alanine	8.0 b	9.5	7.6	1.06	941	41	38,580
Leucine	3.2 b	5.6	4.8	0.42	2,350	17	39,950
Isoleucine	2.2 b	3.4	2.9	0.29	3,420	11	37,620
Valine	1.9 b	4.2	3.7	0.25	3,960	10	39,600
Phenylalanine	0.6 b	1.4	1.3	0.08	12,540	3	37,620
Tyrosine	0.0 d	0.0	0.0	0.00	—	—	—
Tryptophan	2.5 b	3.4	2.7	0.33	3,010	13	39,130
Serine	1.5 b	2.4	2.0	0.20	5,020	8	40,160
Threonine	0.0 e	0.0	0.0	0.00	—	—	—
Cystine	0.4 a	0.8	0.7	0.05	18,820	2	37,640
Methionine	9.9 b	15.1	12.7	1.32	760	51	38,760
Proline	8.0 c	14.0	12.1	1.07	941	41	38,580
Hydroxyproline	4.7 b	4.5	4.0	0.31	3,200	12	38,400
Lysine	1.2 b	1.3	1.1	0.08	12,560	3	37,680
Hydroxylysine	15.3 b	8.8	7.9	0.51	1,969	20	39,380
Arginine	1.2 b	0.8	0.7	0.05	18,820	2	37,640
Histidine	3.6 a	6.3	5.5	0.47	2,092	19	39,750
Aspartic acid	5.8 a	11.3	10.0	0.77	1,297	30	38,910
Glutamic acid	3.5 a	0.66	—	0.47	2,157	18	38,740
Amide N	99.8	119.00*	99.6*	10.76*	—	419	38,730
Total found							(mean)

* Excluding amide N

(a) Determined by present authors

(b) Determined by Chubnall and collaborators on the same sample of collagen.

(c) Bergmann & Stein (1939)

(d) Block & Bolling (1945)

(e) Baernstein (1930)

The values obtained by the present authors for the dicarboxylic acids are 50% higher than the earlier values of Dakin (1920), and, although probably still an underestimate, are the best values at present available for collagen (Consden *et al* (1948) report 91 and 96% recoveries of glutamic and aspartic acids, respectively, from synthetic mixtures simulating wool). If a correction based on the results of Consden *et al* (1948) on wool is applied, the total dicarboxylic acid content agrees well with that deduced from the titration curve.

With the exception of the ϵ amino groups of lysine, the values deduced from the titration curve are in agreement with those obtained by analysis, and there is no indication that any of the other reactive side-chain groups of the basic or dicar-

boxylic acids are bound, or that there is any appreciable number of terminal amino or carboxyl groups. From a survey of the literature, it is evident that of the remaining amino acids, tryptophan and cystine are absent, but that glycine and hydroxyproline are present in considerable amounts. Values for these last two have been selected from the literature to complete the attempted survey of the amino acid composition of collagen given below. Of these, the figure for hydroxyproline is most likely to be in error, the method used is not entirely reliable and the determination was made on gelatin.

When the composition of collagen was considered by Braybrookes (1939) and by Bowes (1943) the analysis was incomplete. The higher figures now obtained for some of the amino acids, notably phenylalanine and the dicarboxylic acids, and the addition of valine, threonine, serine, and hydroxy-

lysine to the list of amino acids present in appreciable amounts, have now made up the deficiency. Using the values given in Table 4, it is now possible to account for over 99% of the total N of collagen (see col 1), and the sum of the amino acid residues (col 3) approaches 100. The analysis of collagen is, therefore, virtually complete. Although the values of some of the amino acids may be adjusted slightly as a result of improved methods of analysis, it is unlikely that any great changes in the values will be made, or that other amino acids will be found to be present in important amounts.

Calculation of the minimum molecular weight from the data in cols 1, 5 and 6 gives a value of about 39,000. Earlier calculations from the composition of gelatin have given figures of 10,300 (Jordan Lloyd, 1920), 10,000–12,700 (Cohn, Hendry & Prentiss, 1925), and 34,500 (Atkin, 1933).

The mean residue weight calculated by summation from the data in Table 4

$$\frac{\Sigma \text{ wt of amino acid residues/100 g protein}}{\Sigma \text{ g residues/100 g protein}}$$

is 92.6, and the same value is obtained by calculation from the N distribution (Chibnall, 1942). From X-ray data and the density of dry gelatin Astbury (1940) obtained a value of 94 for the average residue weight in gelatin. Taking 39,000 as the mean molecular weight and 92.6 as the average residue weight, the number of residues/molecule is 421, of which 419 have been identified (col 6, Table 4).

SUMMARY

1 Collagen has been prepared from ox hide with the minimum of chemical treatment. The total N

and amide N contents of the collagen are higher than those previously reported. The glutamic and aspartic acid contents are nearly double the values quoted by Dakin (1920) for gelatin.

2 The titration curve of this collagen has been interpreted in terms of the titratable groups, and the values deduced are, in general, in agreement with those obtained by direct analysis, and indicate that the side chain groups of the basic and dicarboxylic acids are free, and that the number of terminal groups is small.

3 It is now possible to account for over 99% of the nitrogen in collagen, and it is considered that it is unlikely that any further amino-acids will be found to be present in important amounts.

4 The minimum molecular weight calculated from the analytical figures is 39,000, and the average residue weight calculated both by summation and from the nitrogen distribution is 92.6.

In the course of this and other work the authors have had occasion to refer to the various determinations of the amino acids in gelatin and collagen which have been made at different times, and it has been thought useful to put these on record. Copies of this compilation may be obtained from the Librarian, British Leather Manufacturers' Research Association.

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The Effect of Alkalis on Collagen

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An essential process in the conversion of hides and skins to most types of leather is the removal of the hair or wool by treatment in suspensions of lime and sodium sulphide, usually in the cold, for periods varying from a few hours to several weeks

Although several workers have investigated the effect of mild alkaline treatment on collagen (Marriott, 1928, 1931, Kuntzel & Phillips, 1933, Kubelka & Knödel, 1938, Highberger & Stecker, 1941, Ames, 1944), each has dealt only with one or two of the possible changes which may occur, and in only one instance (Marriott, 1931) has a collagen been used which has had no previous alkaline or enzymic treatment

The present investigation has been carried out, therefore, on a collagen which has received no previous chemical treatment likely to cause chemical modification (see Bowes & Kenten, 1948), with the aim of obtaining more precise information regarding possible changes occurring during mild alkaline treatment

EXPERIMENTAL

Alkali treatment of collagen Collagen prepared as previously described (Bowes & Kenten, 1948) was cut into cubes about 1 cm square, and 100 g of this material, together with 310 ml. of water, 1.55 g of $\text{Ca}(\text{OH})_2$, and 90 ml of N NaOH were incubated in a sealed flask at 20° . A preliminary experiment showed that these quantities gave an initial OH^- concentration of 135 m-equiv/l (pH 13.3 at 20°), and a final value of 68 m-equiv/l (pH 13.0 at 20°). After 14 days the flask was cautiously opened to avoid loss of NH_3 , sufficient HCl added to bring the contents to pH 4.5–5.0, the flask resealed and left at 20° for 3 days. Two batches of collagen (I and II) were treated in this way

The collagen was filtered off, washed in running water for several hours, treated repeatedly with acetic acid pH 3 containing 1.5% NaCl , exhaustively washed with water at pH 8, finally with water at pH 6, then dehydrated with acetone. The pH of the last wash water was 5.5 (A little toluene was added to all wash waters to inhibit the growth of micro organisms)

An appreciable amount of protein matter (solubilized collagen) was precipitated on neutralization. This material was filtered off with the collagen and removed during the washing in running water

The total N, amino N, NH_3 and urea were determined in the solution in which the collagen had been treated. It was assumed that the solution held by the collagen was of the same composition as the external solution, and that the total volume was equal to that of the solutions added plus the water held by the original collagen, and the results were calculated accordingly. The total N, amino N, amide N, arginine, total hydroxy acids (serine + threonine + hydroxylysine), and the titration and swelling curves of the collagen, were also determined. All results are expressed on moisture and ash free collagen. Since the total N of the alkali treated collagen is lower than that of the original collagen, the results expressed as a percentage of the total N may be misleading, and they have therefore been recorded also as mmol./g protein

Analysis Total N, amide N and amino N were determined as previously described (Bowes & Kenten, 1948). Arginine was determined by precipitation with flavianic acid (Vickery, 1940). The value obtained (8.3%) was rather lower than that of 8.8% found for the same collagen sample by Macpherson (1946), who used a colorimetric method following the separation of the basic amino acids by electrodialysis

Hydroxyamino-acids The total hydroxyamino acids (serine + threonine + hydroxylysine) were determined by the periodate method as described by Rees (1946), except that the periodate oxidation and subsequent distillation of the NH_3 were carried out in a micro Kjeldahl apparatus. The following solutions were successively added to the micro Kjeldahl apparatus, while preserving a liquid seal at the ground glass stopper: 5 ml of hydrolysate, 5N NaOH to adjust the pH to 7, N H_2SO_4 (sufficient to give a final pH of 7.0 after addition of other reagents), 1.5 ml. of NaAsO_2 solution, 6 ml. of phosphate buffer pH 7.2, 1 ml. of 0.5M- HIO_4 , 3 ml. water. The contents of the micro Kjeldahl flask were thoroughly mixed after each addition by warming the steam flask, and thereby causing a stream of air bubbles to pass through the solution. The NH_3 (preformed and periodate) was distilled off from 5 ml. of borate buffer pH 12.0. The preformed NH_3 was determined by a separate dis-

tillation of 5 ml of hydrolysate at pH 7.0 from 5 ml of borate buffer

Other determinations Ammonia in solution was determined by addition of excess K_2CO_3 and aerating into standard acid, urea by conversion to NH_3 by the action of urease followed by the same procedure (Van Slyke & Cullen, 1914). The urea NH_3 was then calculated by difference. It was necessary to use twice as much urease as that employed by Van Slyke & Cullen (1914) in order to obtain satisfactory results, this was probably due to inhibition of the urease by the high salt concentration of the solution (cf Conway, 1947). The amount of urea was small compared with that of NH_3 , and tests showed that under the present experimental conditions the method gave an accuracy of $\pm 10\%$.

Titration and swelling curves The titration curve of the alkali treated collagen (batch I) was determined in the presence of 0.5M NaCl as previously described (Bowes & Kenten, 1948). For the swelling curves, 0.5 g samples of air dry collagen were placed in 100 ml of solution containing varying amounts of HCl and NaOH at 20°. After 3 days, a sample of liquid was withdrawn for pH determination, and the pieces of collagen lightly blotted and weighed. The vessels used were the same as those employed for the titration curve, and the same precautions were taken to avoid contamination with CO_2 . The water content, expressed as a percentage of the moisture and ash free collagen, was plotted against the final pH value of the solution

RESULTS

Analytical

There is good agreement between the results on the two separate batches of treated collagen, indicating that the results obtained are reproducible (see Table 1)

Although the treatment given was comparatively drastic, the collagen only decreased in weight by about 5%, and was unchanged in appearance. Approximately half the amide N was liberated during 14 days' treatment, and a corresponding amount of ammonia was found in the solution, there

Table 1 Analyses of collagen, alkali treated collagen and of collagen dissolved by alkaline solution

	Collagen					Solution		
	Un-treated	Alkali treated		Increase or decrease			Batch I	Batch II
		Batch I	Batch II	Batch I	Batch II			
Ash (%)	0.03	0.01	0.10	—	—	—	—	—
Water (%)	22.3	23.7	23.8	—	—	—	—	—
Total N (%)	18.6	18.2	18.1	-0.4	-0.5	Total N (mg/g)	9.0	8.1
Amino N (as % TPN)	2.48	2.77	2.94	—	—	Amino N (mg/g)	4.3	4.3
Amino N (mmol/g)	0.33	0.36	0.38	+0.03	+0.05	—	—	—
Amide N (as % TPN)	3.54	1.69	1.85	—	—	Ammonia N (mg/g)	3.46	3.36
Amide N (mmol/g)	0.47	0.22	0.24	-0.25	-0.23	Ammonia N (mmol/g)	0.25	0.26
Arginine N (as % TPN)	14.45	14.17	14.24	—	—	Urea N (mg/g)	0.45	0.42
Arginine N (mmol/g)	0.48	0.46	0.46	-0.02	-0.02	Urea (mmol/g)	0.016	0.015
Periodate NH_3 (as % TPN)	4.54	4.45	—	—	—	—	—	—
Periodate NH_3 (mmol/g)	0.60	0.58	—	-0.02	—	—	—	—

TPN = total protein nitrogen

was no indication that an appreciable amount of ammonia was derived from any other source. The amount of ammonia formed was almost identical with the maximum value obtained by Highberger & Stecker (1941). Further treatment for 7 days under similar conditions, however, lowered the amide N by another 0.08 mmol to 0.16 mmol/g, with the production of 0.06 mmol of ammonia.

The small decrease in the arginine content is less than that found by Theis & Jacoby (1941) in similar experiments, and the urea found in the solution was also less than that found by Highberger & Stecker (1941) under corresponding conditions. It appears probable that the urea arises from conversion of the arginine to ornithine residues (cf. Hellermann & Stock, 1938; Warner, 1942a), the decrease in arginine

(this small increase could be accounted for by the conversion of arginine to ornithine), and, even allowing for the fact that because of the high proline and hydroxyproline content of collagen one out of every four peptide links involves an imino and not an amino group, the alkaline treatment has apparently caused little breakdown of the polypeptide chains of the remaining insoluble collagen.

Titration curves

At pH values between 5 and 10, a longer time was required to reach equilibrium with alkali-treated collagen than with the original collagen. There was rather more soluble nitrogen at high pH values, and rather less at low pH values than with the original collagen (see Table 2).

Table 2 Nitrogen in solution at different pH values after steeping for 3 days

(Results expressed as mg N/g moisture and ash free collagen.)

Collagen				Alkali treated collagen			
pH (20°)	Soluble N	pH (20°)	Soluble N	pH (20°)	Soluble N	pH (20°)	Soluble N
1.16	0.85	12.86	1.44	1.18	0.48	—	—
1.32	0.77	12.71	1.08	—	—	12.77	2.15
1.51	0.69	12.66	1.21	1.42	0.36	12.69	2.05
1.64	0.70	12.59	1.28	1.82	0.34	12.54	2.26
—	—	12.46	1.04	—	—	12.39	1.26
—	—	—	—	—	—	12.20	1.45

content, however, is rather greater than can be accounted for as urea, indicating some other type of breakdown. The alkali-treated collagen, when tested for the presence of citrulline by the method of Fearon (1939), gave a very faint pink colour, the original none at all.

Within experimental error, the ammonia formed by the action of periodate on the untreated and alkali-treated collagen is the same, indicating a negligible destruction of the hydroxyamino acids by the alkaline treatment. The hydroxyamino acids of the collagen used in this investigation have also been determined by Rees (1946), who obtained a value of 4.57% for the periodate NH_3 (expressed as a percentage of the total protein N), in good agreement with the present determination of 4.54%.

The ammonia and urea in the solution account only for about half of the total N present. The figure for the amino N of the solution is likely to be too high owing to the presence of ammonia (25–30% of which may be returned as amino N (Warner, 1942b; Frost & Heinsen, 1945)), but the results suggest that this soluble N must be present as amino acids or small peptides. It may be observed that the sum of the ammonia and urea N in solution is approximately equal to the decrease in the total N of the collagen.

The amino N of the alkali-treated collagen was only slightly higher than that of the original collagen

The titration curve of the alkali-treated collagen differs in a number of respects from the curve of the original collagen, especially in the alkaline range (see Fig. 1). The isoelectric point is shifted from pH 6.7 to 5, the acid binding is slightly increased, and on the alkaline side of the isoelectric point there is a large increase in the number of groups titrating between pH 5 and 9, and a corresponding increase in the base binding capacity. The large increase in the groups titrating between pH 5 and 9 may be attributed to an increase in the number of carboxyl groups arising from hydrolysis of amide groups.

The curve was analyzed in a manner similar to that of the untreated collagen (see Bowes & Kenten, 1948). It can be divided into four portions, from pH 1.5 to 5, from 5 to 7, from 7 to 9.6 and from 9.6 to 12, and to these different portions are assigned the groups shown in Table 3. Imino groups, due to breakdown of peptide links involving proline or hydroxyproline, are taken as mainly titrating between pH 7 and 9.6.

Assuming that the imidazole groups are unaffected by alkaline treatment (Plummer, 1916), and that they titrate in the same range as in the untreated collagen, it can be deduced that there are 0.14 mmol uncharged carboxyl groups/g, and 0.13 mmol basic groups/g (α amino, imidazole or imino), titrating between the isoelectric point and pH 9.6. The assumption that the imidazole groups

are not affected may not be strictly true, since there is some evidence that gelatin contains less histidine than collagen (Chibnall, 1946). The difference, however, is small, and even if a corresponding decrease in histidine does occur here, it would not materially affect the calculation. The same conclusion is

reached if the increase in acid-binding capacity is taken as due to increase in the number of α amino and imino groups, again assuming that these groups titrate between pH 5 and 9.6, and that the α -amino and imidazole groups present in the original collagen remain unchanged.

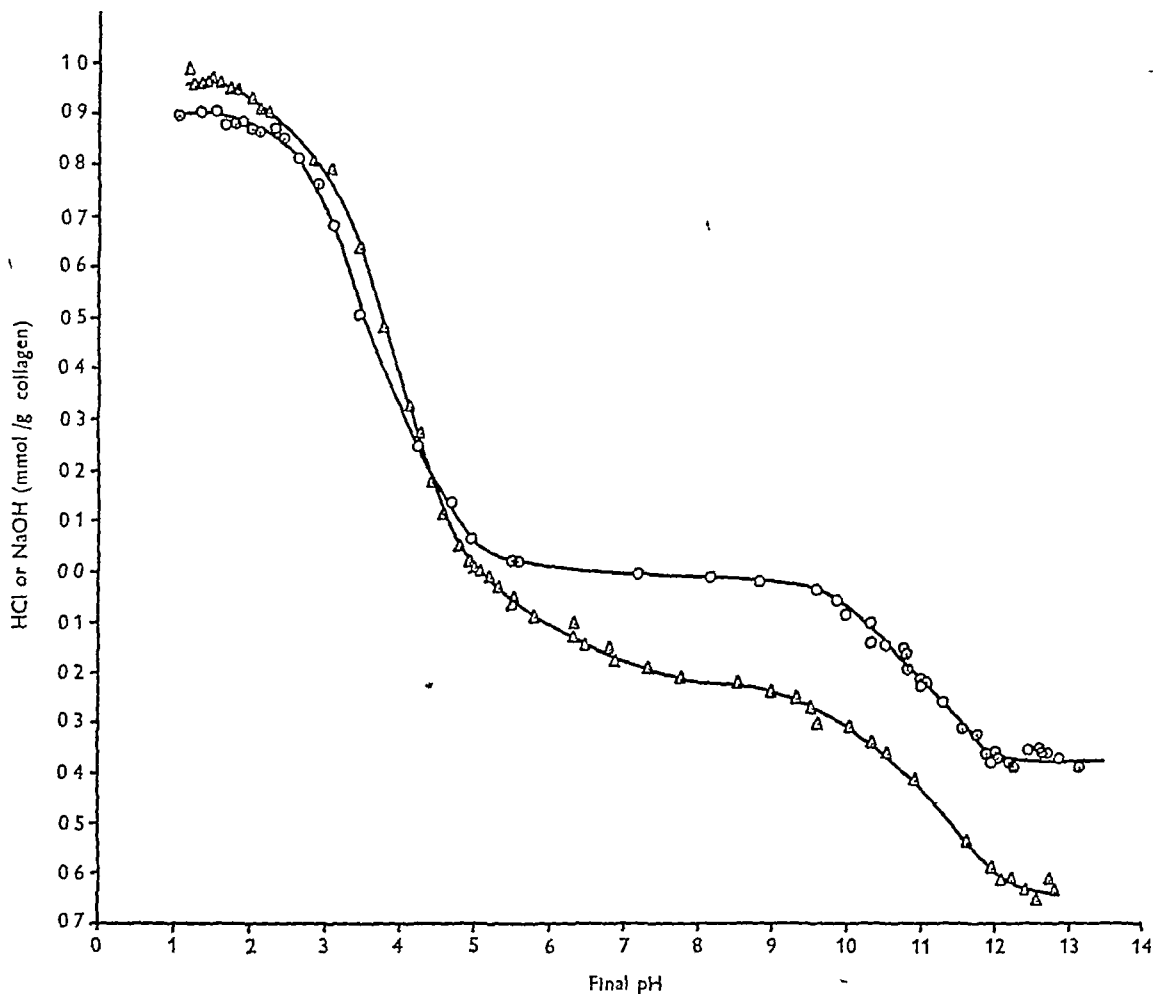


Fig. 1 Titration curves of ox hide collagen before (circles) and after (triangles) treatment with alkali

Table 3 Analyses of titration curves

(Results expressed as mmol/g)		Original collagen	Alkali treated collagen
Group	Method of calculation		
(a) Charged carboxyl	Titration from pH 1.5 to 5	0.87	0.96
(b) Uncharged carboxyl + imidazole	Titration from pH 5 to 7	0.03	0.17
(c) Imidazole, α amino + imino	Titration from pH 7 to 9.6	0.04	0.10
(d) ϵ Amino	Titration from pH 9.6 to 12.5	0.34	0.36
(e) Uncharged carboxyl	(b) - 0.03 mmol. imidazole groups titrating in this range	0	0.14
(f) Total carboxyl	(a) + (e)	0.87	1.10
(g) α Amino + imino	(c) - 0.02 mmol. imidazole groups titrating in this range	0.02	0.08
(h) Guanidino	(a) + (e) - (b) - (c) - (d)	0.49	0.47
(i) Amide	From analysis	0.47	0.22
(j) Dicarboxylic acids	(f) + (i) - (g)*	1.32	1.24

* It is assumed that terminal carboxyl groups are equivalent to the free α amino and imino groups

If these conclusions are correct, there is an apparent decrease in the dicarboxylic acids, which suggests that there may have been specific loss of these amino acids in the fraction of the collagen rendered soluble by the alkaline treatment

The decrease in arginine content calculated from the titration curve agrees well with that found by analysis (Table 1), and the small increase in groups titrating between pH 9.6 and 12 is in accordance with the hypothesis that the arginine is converted to ornithine (pK 10.76) (Cohn & Edsall, 1943). There is no indication of any decrease in the number of ϵ amino groups

Swelling curve

The uptake of water by the collagen at different pH values before and after treatment with alkali

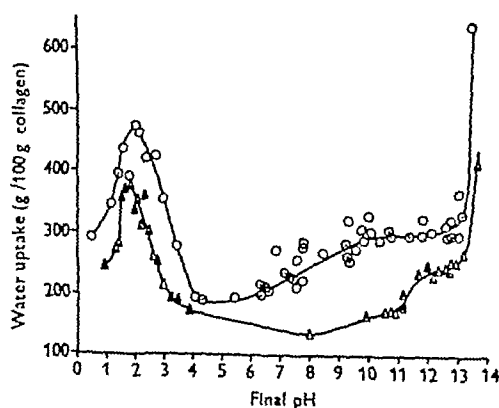


Fig. 2 The water uptake of α hide collagen before (triangles) and after (circles) treatment with alkali

is shown in Fig. 2. After exposure to the acid or alkaline solutions, the pieces of alkali-treated collagen had a roughened appearance especially at

high or low pH values. This was probably an indication of the beginning of disintegration. At all pH values the water uptake is appreciably increased by the alkaline treatment, and on the alkaline side of the isoelectric point the shape of the curve is appreciably different from that of the original collagen.

Analysis of samples of skin collagen after alkaline processing ('liming')

The total N, amino N and amide N were determined in a number of skins which had been treated commercially with calcium hydroxide and sodium sulphide to remove the hair ('limed'). The titration curves and arginine contents of three samples were also determined.

Table 4 shows that, with the exception of the sheep skin collagens, the total N of all the samples was of the same order as that of the alkali-treated α -hide collagen. The amide N values varied from 0.25 to 0.47 mmol/g, the sheep skins on the whole having the lowest values. These skins had been treated with a commercial preparation of trypsin ('bating') and with sulphuric acid (approx. 1%) and sodium chloride ('pickling'), and this is probably the cause of the lower values. In a number of cases, the amino N of the commercially treated samples is lower than that of the ox hide collagen.

The titration curves of sheep skins 1, 2, 3 differ from one another and from the curves for α -hide collagen, especially in the pH range 6-9.

The curves are more difficult to interpret in terms of the reactive groups than those of the ox hide collagen. The various sections of the curve, especially those due to titration of unionized carboxyl, imidazole, α amino and imino groups tend to merge into one another. Treating the curves similarly to that of the alkali-treated collagen, the analyses given in Table 5 were deduced.

Table 4 Total N, amide N and amino N of collagen of some commercially treated skins

Source of collagen	Total N (%)	Amide N		Amino N	
		(%)	(mmol/g)	(%)	(mmol/g)
Ox hide collagen (no alkaline treatment)	18.6	0.66	0.47	0.46	0.33
Sheep skins 1	17.3	0.42	0.30	—	—
2	17.1	0.42	0.30	0.59	0.42
3	17.2	0.35	0.25	0.50	0.36
4	17.4	0.50	0.36	0.45	0.32
5	17.3	0.49	0.35	0.49	0.35
Calf skins 1	18.2	0.56	0.40	—	—
2	18.2	0.52	0.37	—	—
3	18.0	0.55	0.39	0.42	0.30
Ox hide 1	18.1	0.66	0.47	0.46	0.33
2	18.1	0.42	0.30	0.45	0.32
3	18.3	0.46	0.33	0.48	0.34
4	18.1	0.57	0.41	0.42	0.30

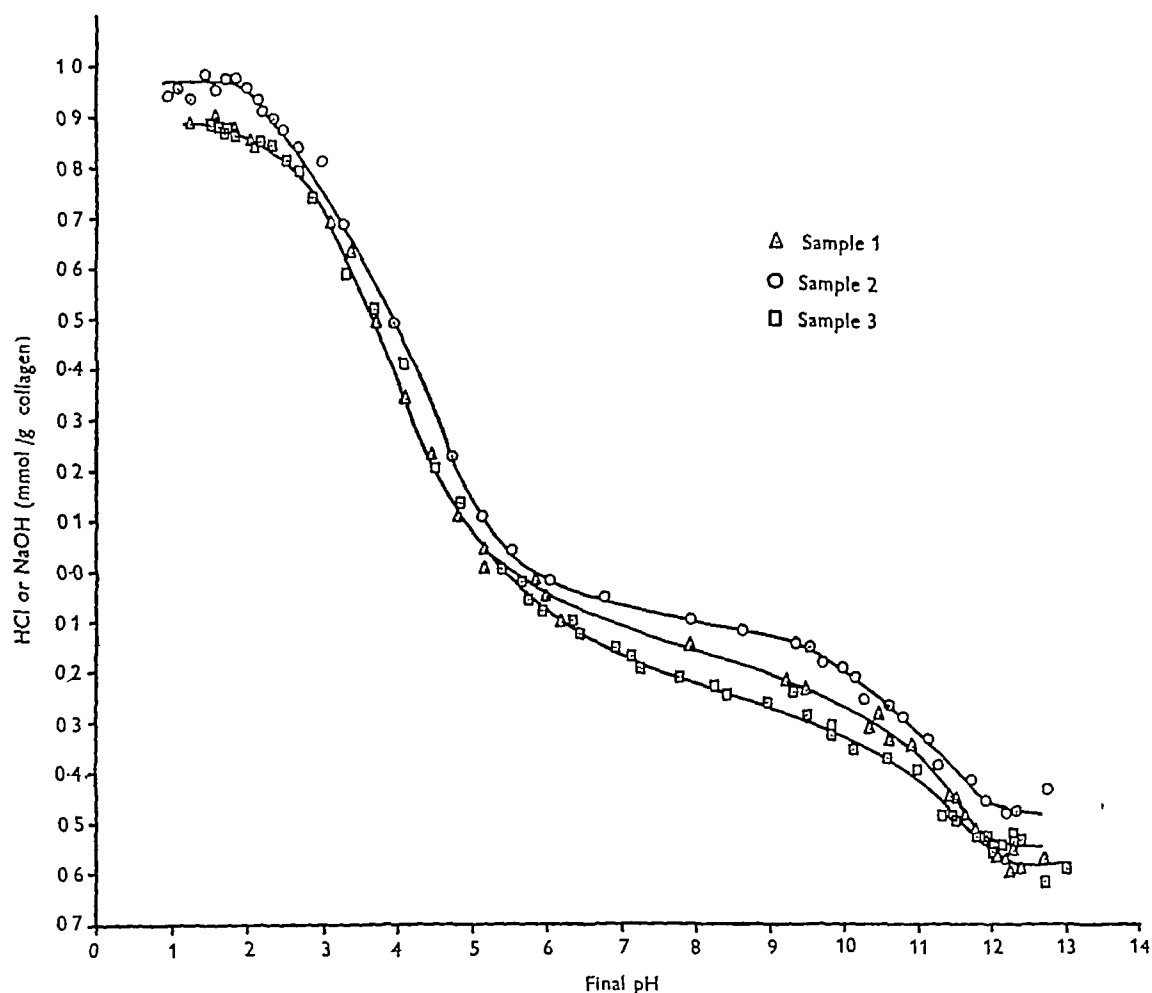


Fig 3 Titration curves of commercially treated sheep skins

Table 5 Analyses of titration curves of alkali-treated sheep-skin collagens

(Results expressed as mmol./g)

Group	Method of calculation	Alkali treated sheep skin collagen		
		Sample 1	Sample 2	Sample 3
(a) Charged carboxyl	Titration from pH 1.5 to isoelectric point	0.89	0.97	0.88
(b) Uncharged carboxyl + imidazole	Titration from isoelectric point to pH 7	0.10	0.08	0.16
(c) Imidazole, α amino + imino	Titration from pH 7 to 9.6	0.14	0.10	0.13
(d) ϵ -Amino	Titration from pH 9.6 to 12.5	0.34	0.32	0.26
(e) Uncharged carboxyl	(b) - 0.03 mmol imidazole groups titrating in this range	0.07	0.03	0.13
(f) Total carboxyl	(a) + (e)	0.96	1.00	1.01
(g) α Amino + imino	(c) - 0.02 mmol. imidazole groups titrating in this range	0.12	0.08	0.11
(h) Guanidino	(a) + (e) - (b) - (c) - (d)	0.38	0.52	0.46
(i) Amide	From analysis	0.30	0.30	0.25
(j) Dicarboxylic acids	(f) + (i) - (g)*	1.14	1.22	1.15

* It is assumed that terminal carboxyl groups are equivalent to terminal α amino and imino groups

DISCUSSION

Considering the comparative severity of the alkaline treatment, the chemical modification of the collagen is comparatively small. Apart from the solubilization of about 5 % of the collagen, the only reaction taking place to any appreciable extent is the hydrolysis of amide groups. Since the loss of amide groups increases with time, it appears probable that all the amide N would be liberated if the treatment were sufficiently prolonged.

The small decrease in arginine content and the presence of urea in solution indicates a breakdown to ornithine, as postulated by Hellermann & Stock (1938) and Warner (1942*a*). A positive test for citrulline in the alkali treated collagen indicates that the conversion of arginine to citrulline and ammonia also takes place to a very small extent (see Warner, 1942*b*). The amount of ammonia arising from arginine is negligible compared with that due to deamidation.

Nicolet, Shinn & Saidel (1942) have shown that treatment of silk fibres with boiling 0.1 M alkali for 1-2 hr causes partial destruction of serine and threonine, producing an approximately equivalent number of amide groups. There is no evidence that such a reaction occurs even to a limited extent when collagen is treated with alkali in the cold for periods up to 14 days.

The ornithine produced by modification of the guanidino groups could account for the small increase in the base bound between pH 9.6 and 12.5, and for the greater part of the increase in free amino N. On this basis, there can be little, if any, hydrolysis of peptide links involving amino groups in the alkali treated collagen, and the increase in acid-binding capacity (0.06 mmol/g) must presumably be due to release of basic groups other than amino groups. It is possible that these are imino groups, derived from hydrolysis of peptide links involving proline or hydroxyproline. Whilst this would suggest that such peptide links are more alkali labile than those involving amino groups, Gordon, Martin & Syngé (1943), from a study of the partial acid hydrolysate of gelatin, inferred that they were resistant to acid hydrolysis at 37°.

Although there is only a small increase in the number of terminal groups in the alkali treated collagen, an appreciable amount (5 %) of the collagen was rendered soluble by the treatment. Some of this was presumably in the form of large polypeptides, since it was precipitated on neutralization of the solution, peptides which did not precipitate probably account for nitrogen in the solution in excess of that due to ammonia and urea. The amino N figure for the solution suggests that this fraction must be in the form of amino acids or small peptides. Evidence deduced from the titration curve suggests that this soluble fraction is rich in

the dicarboxylic acids. Martin (1946) has recently demonstrated the presence of aspartylglutamic, glutamylglutamic, and glutamylaspartic acids in the partial hydrolysate of wool, and if these two dicarboxylic acids also occur together in collagen, breakdown could readily lead to their specific loss.

Consideration of the swelling curves indicates that physical as well as chemical changes take place during the alkaline treatment, for the changes in the curve can only be partially accounted for by changes in the reactive groups. The water uptake in the isoelectric range is increased, the increase in water uptake in acid solutions is greater than corresponds to the increase in basic groups, and the increase between pH 7 and 9 can only partially be accounted for by the titration of uncharged carboxyl groups liberated by hydrolysis of amide groups. The cause of the shift in the pH at which water uptake in alkaline solutions reaches a maximum from 12.5 to 9.5 is not clear.

Examination of the commercially treated skins indicates that similar changes have taken place, the total N and amide N of these were lower than those of the original ox-hide collagen, but the amino N was in some cases lower, instead of higher as might have been expected from the laboratory experiment. It is possible that this may be due to bacterial deamination at some stage in the processing of the skins (Stuart, 1946). The total N of the sheep skins was particularly low, and is not accounted for by the lower values of the amide N and arginine N as compared with the original ox-hide collagen, which suggests the possibility that the composition of sheep skin collagen may differ from that of ox hides.

SUMMARY

1 Hydrolysis of amide groups is the main reaction taking place when collagen is treated with alkali at pH 13.0. A small number of the guanidino groups is converted to ornithine and urea, and a still smaller fraction to citrulline and ammonia. There is a small increase in the total number of basic groups and in the number of free amino groups, it is suggested that some peptide links involving proline and hydroxyproline are broken. The hydroxyaminoacids, serine, threonine and hydroxylysine, are unaffected by the treatments. Approximately 5 % of the collagen is solubilized.

2 Alkali treated collagen swells more than the original collagen.

3 Skins treated commercially with alkaline solutions undergo changes similar to those found in laboratory experiments.

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The Nutrition of the Larva of *Aedes aegypti* Linnaeus

3 LIPID REQUIREMENTS

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In a previous communication (Golberg, De Meillon & Lavoipierre, 1945) we showed that water-extracted yeast residue, supplemented by a number of water-soluble vitamins, constitutes an adequate medium for the growth and metamorphosis of the larva of *Aedes aegypti* Linnaeus. From this starting point our investigations were concerned with the part played by lipids in the nutrition of the mosquito larva.

Considering the ability of insects to transform carbohydrate and protein into fat (evidence summarized by Wigglesworth, 1942), it is surprising to find that many insects are incapable of synthesizing sterols, or at least of producing them in sufficient quantities to cover their needs for growth and particularly for metamorphosis. The need for preformed sterol in the diet of many insects has been conclusively demonstrated. Yet serious consideration does not appear to have been given to the possibility that non-steroid lipids may be capable of fulfilling the same nutritive purpose. For example, lecithin has been employed as a component of diets for *Drosophila* (Bacot & Harden, 1922, Guyénot, 1917, Lafon, 1937), and frequent reference is made in the literature to essential lipid factors present in wheat, wheat germ, cod-liver oil, butter fat, and ethanolic extract of yeast. In two instances, in which

attempts have been made to fractionate the fat and isolate the active agent, the conclusion was reached that cholesterol was the essential constituent (Hobson, 1935, van't Hoog, 1936). Fraenkel & Blewett (1945) found that, in addition to cholesterol, insects of the genus *Ephestia* require linoleic acid and vitamin E.

Gay (1938) and subsequent workers (Fraenkel, Reid & Blewett, 1941, Fraenkel & Blewett, 1943) employed yeast in the growth media used for studying the sterol requirements of insects. Although the proportion of yeast in the dietary solids varied from 5 up to 96%, they, nevertheless, found it necessary to add a further quantity of cholesterol to the diet, the minimum being 1 mg sterol/3 g diet (Fraenkel *et al* 1941). Dried yeast has a fat content of 7% (Newman & Anderson, 1933) of which approximately one third consists of sterols (Smedley-MacLean & Thomas, 1920). It would appear that this level of sterols is inadequate for insects normally feeding on flour or other stored food, particularly when the yeast constitutes only 5% of the artificial diet. In the case of *Aedes* larvae, however, when the whole of the solid portion of the growth medium was composed of yeast, no added sterols or other lipids were needed.

EXPERIMENTAL

Aedes aegypti L. The strain of mosquitoes used was the same as in our previous investigations (De Meillon, Golberg & Lavoipierre, 1945, Golberg *et al* 1945)

Method of obtaining sterile eggs and larvae of Aedes aegypti In order to ensure that the eggs used were not more than 24 hr old, the following procedure was employed gravid females from the insectary were confined, singly, in sterile test tubes, together with 2-3 ml sterile distilled water and incubated at 28° The tubes were examined every morning and the females liberated from those tubes containing eggs The eggs and water were poured with aseptic precautions into a fresh sterile tube, and the water with drawn

Tubes with stranded eggs were now half filled with White's solution (White, 1931), left for 30 min, the fluid removed and the eggs washed three times with sterile distilled water In the final washing a platinum loop was used to dislodge any eggs adhering to the sides of the tube The sterilized eggs were transferred directly to tubes containing an autoclaved solution of glucose (1%) and Tatum's (1939) salt mixture (0.15%), previously adjusted to pH 6.5 Incubation at 28° was continued for 96 hr after the larvae had hatched, and served to detect contaminated batches Those found to be sterile were used forthwith

Preparation of media As a rule each experimental medium consisted of a liquid and a solid component, prepared and sterilized separately as follows The liquid component was invariably prepared fresh and sterilized on the same day by pressure filtration through a pad The sterile filtrate was collected in a special apparatus designed to facilitate rapid distribution of the liquid into tubes containing the sterile solid fraction. In general 10 ml. were measured into each tube Where sodium caseinate was used, it was autoclaved and added separately The solid component was sterilized by autoclaving for 30 min. at 15 lb pressure A quantity of 20-50 mg per tube was adequate, but observation of the larva was facilitated by adding an amount sufficient to fill the rounded portion of the tube Lipids were introduced into the solid fraction of the medium by solution in a small volume of pure ether (or other suitable volatile solvent) and stirring with powdered cellulose or kaolin until the solvent had evaporated Such mixtures were autoclaved in the dry state

Media were tested for sterility in the usual manner

Observations of development and methods of recording them The stage of development reached by each larva was recorded every morning and evening, and the time taken for the change from one stage to the next was calculated to the nearest half day In considering the results of experiments, it was thus possible to compare the times taken for each stage ('growth rates') and also the number of larvae reaching that stage ('survival rates') It is realized that the growth rates recorded are subject to a maximum error of +0.5 day, but this does not affect the standard deviations or the significance of differences between groups

In most tables the results have been condensed, but all the data are available in the same detail as in Tables 1 and 2

Materials and composition of media

The basal medium (LM) contained glucose (10 g/l.), salt mixture (Tatum, 1939) (1.5 g/l) and the following vitamins (in mg/l) aneurin chloride, 10, riboflavin, 20, pyridoxin hydrochloride, 10, calcium D pantothenate, 10, nicotinic acid, 10 The pH was adjusted to 6.3-6.5

For use with sodium caseinate the basal medium was prepared in double strength and 5 ml filtered medium added to 5 ml. autoclaved sodium caseinate

Sodium caseinate was at first prepared from purified casein. Later, S.M.A. Co 'Vitamin test' casein was used, and the pH adjusted to 6.6 Immediate autoclaving at 18 lb for 45 min was essential to prevent contamination Analysis showed the average concentration of sodium caseinate in the final medium to be 0.95% (w/v)

Yeast autolysate Difco 'Bacto yeast extract' was used in a concentration of 2% (w/v)

Yeast residues were prepared from fresh brewer's yeast which was dehydrated with ethanol, extracted at room temperature for 24 hr with ethanol, followed by a mixture of ethanol and light petroleum (1:1) After five additional extractions with boiling mixtures of ethanol and benzene (2:1) (Bills, Massengale & Prickett, 1930) it was dried in air, powdered and sieved

Newman & Anderson (1933) showed that treatment with ethanolic hydrogen chloride was necessary for the extraction of a small quantity of yeast fat which cannot be removed by neutral solvents Accordingly, the yeast residue as prepared above was incubated for 2 days at 37° with 1% (w/w) ethanolic hydrogen chloride, after which it was washed and dried as before

Yeast or yeast residue was freed from water soluble constituents by repeated extraction with boiling water and finally by continuous extraction with boiling water in a Soxhlet apparatus for 48 hr

Lipids Freshly prepared and purified egg yolk lecithin and beef brain cephalin were submitted to additional purification as described by Weil Malherbe & Dickens (1944) Careful testing failed to reveal the presence of sterols in the purified materials Sphingomyelin (beef brain) was purified by the procedure of Levene (1916) Kerasin was prepared from the fresh spleen of a case of Gaucher's disease, supplied by Dr J. A. Murray of this Institute

Steroids Many of the sterols and derivatives used were prepared by standard procedures The remainder, including sex hormones and related compounds, were obtained from Drs F. W. Fox and J. Gillman, Prof. I. M. Heilbron and Dr E. R. H. Jones, Mr F. A. Robinson (Glaxo Laboratories Ltd.), Prof. L. Ruzicka and Pl. A. Plattner, Dr E. Schwenk (Research Division, Schering Corporation) and Dr H. A. Shapiro

All steroids used were checked for purity and where necessary submitted to further purification. Only in the case of 7 dehydrocholesterol was the test material of doubtful purity

Methyl linoleate and linolenate were obtained from Prof. T. P. Hilditch

Folic acid A concentrate of the ammonium salt of folic acid, of potency 5000, obtained from Prof. R. J. Williams, was used in a concentration of 20 mg/l. of medium

Pteroylglutamic acid was made available to us by Dr E. L. R. Stokstad (Lederle Laboratories) It was used in a concentration of 25 mg/l.

RESULTS

The growth-promoting properties of fat free yeast and yeast lipids

The basal medium (*LM*) containing glucose, salts, aneurin, riboflavin, pyridoxin, nicotinic and pantothenic acids, when combined with fresh brewer's yeast, produced excellent growth of the larvae. Gradual removal of the fats from the yeast decreased the rate of growth and the number of pupae and adult mosquitoes. Finally, after extraction of the yeast residue with ethanolic hydrogen chloride, there was complete failure to emerge from the pupal stage (Table 1). Recombination of the final yeast residue with the yeast lipids led to normal growth and survival rates, provided that the lipid concentration did not exceed 4%. At 8% lipid concentration the medium was very toxic.

Further experiments were designed to supplement fat free yeast residue with yeast lipid fractions and with a variety of lipids from other sources. The crude yeast fat was able to produce normal growth. Its ether soluble fraction did the same. Of the other fractions tested, that obtained by steam-distilling yeast fat was remarkably toxic. In a concentration of 0.15% on the solid component of the medium it completely prevented growth of the larvae. At higher concentrations it killed them within 24 hr.

Admixture of fat-free yeast residue with cholesterol, purified ovoidlecithin or beef-brain cephalin in each case resulted in growth to the adult stage. The best results were obtained by the use of all three lipids in a total concentration of 2.5%, resulting in growth and survival rates closely similar to those observed with yeast fat.

Table 1 *Rates of growth and survival of mosquito larvae on media containing fat free yeast*

(Here and in subsequent tables No = number in stage of development indicated. The number of first-instar larvae taken was approximately 20 per experiment. Mean, S.D. = mean and standard deviation of time (in days) taken to change from previous stage of development.)

Medium	Larval instars								Adults		
	II		III		IV		Pupae		Total time taken		
	Mean, S.D.		Mean, S.D.		Mean, S.D.		Mean, S.D.		Mean, S.D.		
	No		No		No		No		No	Range	
<i>LM</i> , with additions as indicated											
Fresh brewer's yeast	63	1.9±0.36	63	1.1±0.19	63	1.1±0.29	60	2.6±0.50	57	7.5-11.5	8.8±0.60
Fat-free yeast residue	48	2.6±0.48	48	2.1±0.57	48	3.2±1.03	17	10.7±2.36	0	—	—
Fat-free yeast + yeast lipids (2.5%)	23	2.2±0.29	22	1.3±0.41	22	1.6±0.38	20	3.0±0.62	18	8.5-9.5	9.1±0.49
Water extracted fat-free yeast	7	4.1±1.60	7	2.9±0.83	0	—	—	—	—	—	—
Water extracted fat-free yeast + folic acid	18	1.9±0.99	16	1.9±0.75	1	3.0	—	—	—	—	—
Fat-free water extracted yeast	22	3.8±0.86	20	5.0±1.65	11	5.4±1.09	0	—	—	—	—
Fat-free water-extracted yeast + pteroylglutamic acid	21	1.9±0.63	21	1.6±0.84	12	3.8±3.61	2	5.8	0	—	—
Fat-free water-extracted yeast + yeast autolysate	17	5.0±3.08	16	1.4±0.45	14	3.2±1.96	1	15.0	0	—	—
Fat-free yeast + cholesterol	20	2.7±0.92	20	1.4±0.54	20	1.7±0.55	17	3.9±0.62	16	9.5-15.5	11.6±1.60
Fat free yeast + lecithin	19	2.8±0.88	19	1.9±0.93	17	2.5±1.32	16	3.8±0.50	16	9.5-16.5	12.6±2.10
Fat-free yeast + cephalin	22	2.9±0.98	20	2.3±0.67	20	2.8±1.48	14	5.5±1.44	12	11.5-20	15.1±2.73
Fat-free yeast + cholesterol, lecithin and cephalin (1:1:1)	19	1.6±0.16	19	1.2±0.31	19	1.3±0.49	19	3.4±0.55	19	8.5-10.5	9.7±0.85

Exhaustive extraction of fat-free yeast with water rendered it incapable of supporting growth, even to the fourth larval instar. The addition of folic acid to the medium made no essential difference. Reversal of the order of extraction, using water first and fat solvents later, yielded a residue which promoted better growth and afforded clear evidence of the effect of lipid deficiency on growth and survival of mosquito larvae (Table 1).

Growth on other fat free media

A study was made of the growth of larvae on fat free media not containing solid yeast. Casein, as the source of protein, gave best results when present in solution as the sodium salt. By incorporating yeast autolysate in the medium it was found that the fat free yeast residue could be dispensed with (Table 2). In none of our experimental media could a need for

solid material be demonstrated. However, it was convenient to use a solid in finely divided form as a means of introducing lipids into the medium, and for this purpose cellulose, cellulose acetate or kaolin were equally effective.

Fatty acids and triglycerides

Since a number of compounds seemed capable of bringing about the lipid effect, it was deemed advisable to make sure that this property did not

Table 2 Rate of growth and survival of mosquito larvae on media not containing fat free yeast residue*

Medium	Larval instars						Adults				
	II		III		IV		Pupae		Total time taken		
	No	Mean, s d	No	Mean, s d	No	Mean, s d	No	Mean, s d	No	Range	Mean, s d
<i>LM</i> + yeast autolysate, with additions as indicated											
Casein	36	2.7±1.41	35	2.2±0.78	16	5.1±4.37	0	—	—	—	—
Sodium caseinate	20	3.1±0.33	20	1.4±0.39	18	2.4±1.42	8	5.9±1.19	0	—	—
Sodium caseinate + cellulose	21	1.9±0.37	20	1.6±0.21	16	3.0±1.44	3	7.0	1	—	12.5
Sodium caseinate + kaolin	34	1.8±0.63	34	1.9±0.48	25	3.2±1.85	7	6.5±0.76	3	12.5–15	14.0
<i>LM</i> + yeast autolysate + sodium caseinate + lipids in 2.5% concentration on cellulose											
Cholesterol	17	2.0±0.51	17	1.3±0.24	17	1.8±0.25	13	3.8±0.75	9	9.5–12.5	11.0±1.04
Lecithin	24	1.8±0.38	24	1.4±0.36	24	1.9±0.39	22	4.4±1.14	16	9.5–14.5	11.9±1.49
Kephalin	25	1.7±0.32	25	1.6±0.42	24	2.4±1.36	8	5.3±2.10	1	—	10.0
Cholesterol + lecithin + kephalin (1 2 1)	40	2.1±0.67	40	1.5±0.42	39	1.9±0.51	34	3.4±0.65	32	9.5–13	10.8±1.09
Sphingomyelin	17	2.4±0.78	17	1.2±0.60	17	1.7±0.69	11	3.9±0.63	7	9.5–11	10.5±0.60
Keratin	22	2.3±0.89	22	1.3±0.42	22	1.8±0.38	18	3.8±0.65	16	10.5–13	11.4±0.92
β Carotene	44	3.3±0.76	44	2.0±0.60	44	2.8±1.48	31	6.2±2.10	23	11–29	16.9±2.81
α Tocopherol	37	2.3±0.39	37	2.2±0.61	30	3.4±0.81	15	8.3±2.48	11	13–23.5	16.8±2.11
α Tocopheryl acetate	15	2.4±0.60	15	2.0±0.45	15	2.7±0.69	12	8.4±2.63	8	12.5–24.5	17.5±3.08

* For explanation of headings see Table 1

Table 3 The effect of cholesteryl esters on growth and metamorphosis of mosquito larvae

(Medium *LM* + sodium caseinate + yeast autolysate + cholesteryl ester (2.5 %) on cellulose)

Cholesteryl ester tested	No of second larval instars	No of adult mosquitoes	Total time taken	
			Mean	s d
Unesterified	22	11	11.8 ± 0.97	
Acetate	23	14	10.6 ± 0.69	
Palmitate	17	12	13.5 ± 1.74	
Stearate	20	11	13.4 ± 2.02	
Oleate	14	5	12.3 ± 1.29	
Cerotate	18	6	13.7 ± 1.32	
Arachidate	17	9	16.9 ± 4.36	
p Aminobenzoate	17	15	18.0 ± 1.90	
Nicotinate	19	15	12.4 ± 2.37	

A medium was thus available for testing the effects of various lipids. Cholesterol and lecithin were capable of bringing about pupation of the larvae and emergence of adults, but cephalin was totally ineffective. On combining these lipids in pairs, or using all three in various proportions, the rates of growth were but little affected, while the rates of survival varied considerably. The highest proportion of adult mosquitoes (80 %) resulted from the use of lecithin, cholesterol and cephalin in the proportion of 2:1:1. This mixture was adopted as the standard source of lipids for subsequent 'synthetic' growth media.

Of the other lipids tested under the same conditions, only keratin and, to a lesser extent, sphingomyelin, β carotene and α tocopheryl acetate, were capable of replacing cholesterol.

extend to the common fatty acids and triglycerides. None of the following could replace cholesterol: palmitic, stearic and oleic acids, tripalmitin, tristearin and triolein. Lauric and myristic acids were highly toxic, so too was oleic acid, even in a concentration of 0.5 % on kaolin. More highly unsaturated fatty acids—linoleic and linolenic acids, even when used with α tocopherol (Fraenkel & Blewett, 1945) were inactive. Furthermore, all the above mentioned compounds proved incapable of supplementing cholesterol in the way in which lecithin and cephalin had done. Similarly, esterification of cholesterol with acetic, oleic, palmitic, stearic, cerotic and arachidic acids had no striking beneficial effect on larval growth and survival (Table 3).

Lipids in solution

On testing the effect of presenting the lipids in solution, it was found that neither lecithin nor cholesterol had any apparent effect. Nor was lecithin in solution able to improve the larval response to cholesterol present in the solid state (Table 4). Fatty acids in solution as the sodium salts were tested under the same conditions: palmitate and stearate had no effect and oleate displayed no toxicity. The concentrations tested were of necessity rather low (0.25%)

results given in Table 5 indicate considerable variation in this respect. In fact, the principal sterols of yeast, when provided in pure form, appear incapable of utilization by the mosquito larva. However, under the conditions of these experiments no sterol—not even cholesterol—produced a wholly satisfactory response, particularly with regard to the proportion of larvae reaching the adult stage. In order to render the medium more fully adequate for the promotion of optimal growth and survival, the powdered cellulose was replaced by a water extracted yeast residue from which all lipids soluble in neutral

Table 4 *The effect of lipids in solution on growth and metamorphosis of mosquito larvae*

(Medium LM + sodium caseinate + yeast autolysate)

Lipid in solution	Concentration (g/l)	Lipid on cellulose (4% concentration)	No. of second larval instars	No. of adult mosquitoes	Total time taken	
					Mean	S.D.
None	—	None	28	2	13.0	
Lecithin	0.4	None	69	5	14.5	
Lecithin	0.25	None	15	2	13.0	
Lecithin	0.4	Cholesterol	23	10	10.9 ± 0.87	
Lecithin	0.25	Cholesterol + cephalin (1:1)	63	38	12.5 ± 0.99	
Lecithin	0.4	Cephalin	18	1	10.5	
None	—	Lecithin	24	16	11.9 ± 1.49	
Cholesterol	0.05	None	23	1	18.5	

Table 5 *The effects of various sterols on growth and metamorphosis of mosquito larvae*

(Medium LM + sodium caseinate + yeast autolysate + sterol (2.5%) on cellulose)

Sterol tested	No. of second instar larvae	No. of adult mosquitoes	Total time taken	
			Mean	S.D.
Cholesterol	22	11	11.8 ± 0.97	
Cholesterol (purified through the dibromide)	39	15	11.5 ± 1.53	
Coprosterol	22	10	13.5 ± 1.87	
β Sitosterol	43	32	10.7 ± 1.12	
Stigmasterol (acetate)	23	10	10.6 ± 0.59	
Ergosterol	21	7	9.2 ± 0.84	
Zymosterol	21	1	10.0	

Table 6 *The ability of various sterols to replace cholesterol in a medium containing fat free water extracted yeast residue*

(Medium LM + sodium caseinate + yeast autolysate + sterol (2.5%) on fat-free water extracted yeast)

Sterol tested	No. of second instar larvae	No. of adult mosquitoes	Total time taken	
			Mean	S.D.
Cholesterol	20	19	9.4 ± 0.96	
Coprosterol	19	19	12.5 ± 1.80	
β Sitosterol	15	14	9.5 ± 1.30	
Stigmasterol (acetate)	20	20	8.8 ± 0.73	
Ergosterol	18	12	9.4 ± 0.84	
Zymosterol	19	16	14.1 ± 2.56	
None	17	0	—	

The ability of other sterols to replace cholesterol in the larval diet

It is most unlikely that cholesterol is normally available to the mosquito larva in its natural habitat. Hence the question arises: to what extent can other sterols replace cholesterol in the larval diet? The

organic solvents had been removed. This preparation still contained a small amount of lipid extractable by incubation with ethanolic hydrogen chloride. The residual lipid did not prevent a complete failure to pupate in the absence of added sterol, yet it ensured that when cholesterol was added excellent growth and survival resulted (Table 6). Under these

conditions the plant sterols were fully equal to cholesterol in their effects. The mycosterols were definitely inferior to the others, zymosterol producing a poor rate of growth, ergosterol a low proportion of adults.

Modifications of sterol structure

These results made it desirable to inquire into the effects of modifications in the sterol structure, and to investigate the relationship of the activity of such sterol derivatives to other dietary factors. For this purpose each compound was tested on three solid components in the usual liquid medium. The solids used were cellulose, fat free water extracted yeast, and the yeast residue prior to extraction with ethanolic hydrogen chloride. The conclusions to be drawn from the results, some of which are given in Table 7, may be summarized thus: (a) none of the

pounds of the pregnane, androstane and oestrane series Δ^5 pregnen 3 α -20 one, dehydroisandrosterone acetate, progesterone, deoxycorticosterone acetate, 17 ethynyl- and 17 methyl testosterone, testosterone itself and its propionate, Δ^4 androstene 3 17 dione, pregnane 3(α) 20 α diol, *allopregnane* 3(α) 20 α diol, androsterone, *allopregnane* 3 20-dione, oestrone and oestradiol benzoate, (d) among the inactive compounds were some which were markedly growth inhibitory (marked with an asterisk above)

DISCUSSION

In the experiments described above, the progress of each individual larva was recorded. In consequence it was possible to demonstrate that a lipid-free medium supports larval growth, albeit at a somewhat reduced rate, to the fourth instar. The effect of lipid deficiency reveals itself most frequently in a failure

Table 7 *The growth promoting properties of sterol derivatives*

(Media: LM + sodium caseinate + yeast autolysate + test compound (2.5%) on A, cellulose, B, yeast extracted with neutral organic solvents and water, C, yeast B further extracted with ethanolic hydrogen chloride)

Sterol derivatives tested	No of second instar larvae	No of fourth instar larvae	No of adult mosquitoes	Total time taken
				Mean \pm SD
β Cholestanol on A	22	14	6	12.4 \pm 0.61
B	18	17	12	12.8 \pm 1.04
C	17	17	12	12.5 \pm 1.77
Cholesterol dibromide on A	19	19	9	11.7 \pm 1.05
B	22	22	21	13.0 \pm 2.86
C	20	19	19	12.3 \pm 1.31
3(β) Hydroxy 6 ketocholestane on A	16	12	0	—
B	18	17	9	13.6 \pm 2.07
C	18	16	7	14.9 \pm 1.82
Δ^4 3(β) 6 Dihydroxycholestene on A	21	13	0	—
B	22	22	20	12.1 \pm 1.58
C	19	17	14	12.8 \pm 1.93
7 Dehydrocholesterol on A	21	18	1	12.0
B	17	17	14	12.5 \pm 1.60
C	18	18	17	11.8 \pm 1.27

compounds tested showed any striking degree of activity on cellulose, most were completely inactive, (b) in the presence of both types of yeast residue the following compounds displayed activity: β -cholestanol, cholesterol dibromide, Δ^4 3(β) 6 dihydroxycholestene, 3(β) hydroxy 6 ketocholestane. There was remarkable correspondence between the growth and survival rates on the two types of yeast residue, (c) all other compounds tested proved inactive. These included the following cholesterol derivatives: Δ^4 cholestenone* and its enol acetate*, α -7 hydroxy- and 7 ketocholesterols, Δ^4 3 ketocholestadiene*, $\Delta^{3,5}$ 7 ketocholestadiene*, 3(β) 5 6 (*trans*) trihydroxycholestane*, *cis* and *trans* Δ^5 cholestene 3 4 diols and their diacetates, and methyl Δ^5 3 acetoxybismorcholenate. Calciferol was inactive, as well as the following steroid hormones and related com-

to undergo metamorphosis. In those few instances in which pupation does occur, it takes place after a considerable delay, and only a small proportion of adults emerge.

Lipid deficiency can be corrected, but not completely, by cholesterol or lecithin, kersin and, to a lesser degree, by other lipids. In addition, the action of lipids can be supplementary, and a mixture of cholesterol, lecithin and cephalin gives better results than either cholesterol or lecithin alone (cephalin alone is inactive). There are thus two aspects of lipid action to be considered, which may be referred to briefly as replacement and supplementation of cholesterol.

The ability of lecithin to replace cholesterol cannot be attributed to the presence in it of sterol as an impurity (see p. 373). Also the activity of lecithin

does not appear to be due to component unsaturated fatty acids, since such fatty acids proved incapable of replacing or supplementing cholesterol. This, taken in conjunction with the fact that relatively few steroid derivatives evince this type of activity, leads to the conclusion that the specialized nutritive power is a function of special types of molecules. It is impossible to state at this stage what these molecules have in common. There is not a physicochemical influence on the external medium of the mosquito larva since, when present in solution in the medium, they are inactive. On the other hand, there is a definite parallelism between their effects on the mosquito larva and their ability to overcome the growth inhibition of Gram-positive bacteria induced by linoleic acid. This effect Kodicek & Worden (1945) have attributed to surface activity.

Supplementation of cholesterol is brought about most effectively by fat-free yeast residue. However, attempts to isolate a specific supplementary factor from the yeast residue have failed. One is reminded of the animal dietary factor found necessary by Dorée & Gardner (1938) and Rosenheim & Webster (1941) for the conversion of cholesterol to coprosterol and for analogous metabolic changes. It is suggestive too that some cholesterol derivatives which are inactive when tested alone evince a measure of activity in the presence of the yeast factor.

While some sterols are capable of replacing cholesterol in the diet of the mosquito larva, on the whole little modification of sterol structure is tolerated. This statement is borne out by the findings of other workers who have studied the ability of insects to utilize sterols and cholesterol derivatives (Fraenkel *et al.* 1941, Fraenkel & Blewett, 1943, van't Hoog, 1936). The paucity of the information available makes it all the more regrettable that Fraenkel & Blewett (1943) used impure cholesterol derivatives in their investigations. The most exhaustive study of this type was made by Cailleau (1937) on the ability of sterols to act as growth factors for *Trichomonas columbae*. Although her results are not necessarily applicable to insects, it is interesting that in most instances the ability of the flagellate to utilize sterols, and particularly cholesterol derivatives, runs strictly parallel to that of the mosquito larva. The outstanding differences lie in coprosterol and stigmasterol, which Cailleau found to be inactive.

Finally, it had been hoped by means of these studies to throw some light on the possible metabolism of cholesterol in the mosquito larva. Δ^4 -3,6-Dihydroxycholestene and 3-hydroxy-6-ketocholestane are the only two cholesterol transformation products which are active for the mosquito larva,

and at the same time may be regarded as likely to occur in normal cells during life (Haslewood, 1944). Even these are active only in the presence of the yeast factor. The conversion of cholesterol to coprosterol takes place through the intermediate stage of Δ^4 -cholestenone (Schoenheimer, Rittenberg & Graff, 1935, Anchel & Schoenheimer, 1938, Marker, Wittbecker, Wagner & Turner, 1942). Turfitt (1944, 1946) demonstrated the microbiological conversion of cholesterol to Δ^4 cholestenone. In the mosquito larva Δ^4 -cholestenone is not only inactive but also strongly inhibitory, conversion to the enol acetate does not abolish the inhibitory effect. In keeping with these observations is the lack of activity of *cis* Δ^5 -cholestene 3,4 diol, which Rosenheim & Starling (1937) found to be readily dehydrated to cholestenone by mild acid treatment.

SUMMARY

1 A study has been made of the part played by lipids in the nutrition of the mosquito larva. The lipids were mixed intimately with an inert solid such as cellulose or kaolin or with fat-free yeast residue and introduced into a basal liquid medium.

2 It was found that in a fat-free medium the rates of growth and survival of the mosquito larva do not fall far short of normal. There are, however, few pupae and very few adult mosquitoes.

3 The larval need for lipid may be supplied by cholesterol or ovoidicithin and, less successfully, by a number of other lipids. Of all those tested, no single compound, and no cholesterol ester, was capable of producing normal growth and survival unless fat-free yeast residue was included in the growth medium. Mixtures of lipids were more effective.

4 The following compounds proved inactive: saturated and unsaturated fatty acids, triglycerides, lecithin and cholesterol *in solution* in the growth medium.

5 A number of steroids, including cholesterol derivatives, were tested in the presence or absence of fat-free yeast residue. All but four were inactive.

6 The need for special lipids appears to be common to many insects and lower organisms and to be capable of being satisfied only by a limited number of compounds. The possibility exists that the effect exerted by these compounds is physicochemical rather than nutritional.

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The Nutrition of the Larva of *Aedes aegypti* Linnaeus

4 PROTEIN AND AMINO ACID REQUIREMENTS

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The state of confusion which exists in the literature on the protein and amino acid requirements of insects may be attributed to two causes failure to maintain strictly sterile conditions and failure to ensure adequacy of the medium with respect to all factors other than protein. In turning our attention to the mosquito larva's needs for protein and amino acids we have sought to avoid both pitfalls and can claim to have succeeded with regard to the first. As regards the second, our efforts have not yet culminated in a medium composed of purified individual chemical substances which could produce growth equal to that attained with the original fresh brewer's yeast medium (De Meillon, Golberg & Lavoipierre, 1945). Until that stage is reached, a final answer to some of the questions raised in this paper cannot be given. Meanwhile, it should be emphasized that growth on the media used fell short of what had been produced in the presence of yeast residue. Not only were the growth and survival rates somewhat poorer, but it was obvious that the spontaneity and uniformity which had characterized the response to whole yeast was lacking.

EXPERIMENTAL

Methods The methods used were the same as in our previous investigations (Golberg & De Meillon, 1947). The microbiological estimation of L-valine was carried out by the method of McMahan & Snell (1944).

Media The basal medium (CM) consisted of two components (i) a solid component made up of 0.25 g of a lipid mixture (lecithin, cholesterol and cephalin in the proportions 2:1:1) intimately mixed with 10 g of finely powdered cellulose, and sterilized by autoclaving for 30 min at 15 lb pressure, and (ii) a liquid component made up of two halves one half, which was sterilized by filtration through a pad, contained glucose (10 g/l), yeast autolysate (20 g/l), Tatum's (1941) salt mixture (1.5 g/l) and vitamins (aneurin chloride, 10 mg/l, riboflavin, 20 mg/l, pyridoxin hydrochloride, 10 mg/l, calcium D pantothenate, 10 mg/l, and nicotinic acid, 10 mg/l). The other half of the liquid component, which was sterilized by autoclaving for 45 min at 18 lb pressure, contained the protein in solution.

Amino-acid mixtures were added to the first half of the liquid component prior to filtration. In all instances sterilization of the medium followed its preparation with the shortest possible interval for it was found that even slight contamination incidental to keeping a protein or amino acid

Table 1 *Amino-acids of media*

	(g/l)									
	Amino acid mixtures									
	X	Y	B	C	D	I	N	O	P	R
Glycine	—	—	1.4	1.4	1.4	2.0	1.4	2.0	2.0	2.0
DL-Alanine	—	—	0.5	0.6	0.6	0.2	0.6	0.6	—	—
DL-Valine	0.4	0.8	0.2	0.8	0.8	0.8	0.8	0.8	1.0	—
L-Leucine	—	0.8	0.2	0.8	0.8	0.8	0.8	1.0	1.0	1.0
DL-Isoleucine	0.8	0.8	0.4	0.8	0.8	0.8	0.4	0.6	1.0	1.0
DL-Proline	—	—	0.6	0.6	0.6	0.2	—	—	—	—
L-Hydroxyproline	—	—	0.2	0.2	0.2	0.2	—	—	—	—
DL-Phenylalanine	—	0.6	0.2	0.6	0.6	0.6	0.6	0.6	0.6	—
DL-Glutamic acid	—	—	0.3	0.4	—	—	—	—	—	—
DL-Aspartic acid	—	—	0.2	0.4	—	—	—	—	—	—
DL-Serine	—	—	0.2	0.2	0.2	0.2	—	—	—	—
L-Tyrosine	0.4	0.8	0.2	0.8	0.8	0.4	0.8	0.8	0.8	0.8
L-Histidine HCl (hydrate)	—	0.4	0.2	0.4	0.4	0.8	0.4	0.4	0.8	0.8
L-Arginine HCl	—	—	0.5	0.6	0.6	0.8	0.6	0.6	0.8	1.0
L-Lysine HCl	—	0.4	0.4	0.4	0.4	0.8	0.4	0.8	0.8	0.8
L-Tryptophan	0.4	0.4	0.2	0.4	0.4	0.8	0.4	0.6	0.8	0.8
DL-Threonine	0.4	0.6	0.2	0.6	0.6	0.8	0.4	0.6	1.0	1.0
DL-Methionine	—	0.6	0.4	0.6	0.6*	0.8	0.6	0.6	0.6	1.0
L-Cystine	0.4	0.02	0.2	0.02	0.02	0.02	0.02	0.06	0.04	0.02
Total concentration of amino acids (%)	0.28	0.62	0.67	1.06	0.98	1.10	0.82	1.01	1.12	1.02

* Later increased to 1.0 g/l.

solution might result in extraneous growth stimulating effects on the larvae

The compositions of amino acid mixtures are shown in Table 1

Materials Sodium caseinate was prepared from S.M.A. Co 'Vitamin test' casein. Gelatin was Eastman Kodak purified calf skin gelatin. Other materials used were prepared and purified by standard procedures

L-3,5-Diiodotyrosine was provided by Prof. C. R. Harrington and pteroylglutamic acid by Dr. E. L. R. Stokstad, Lederle Laboratories

RESULTS

Protein requirements of the mosquito larva

In media lacking protein or amino-acids, but complete in other respects, the mosquito larva is incapable of reaching even the second instar. Accordingly, its growth response to added protein or amino acid mixtures is a sensitive index of the extent to which its exacting nutritional needs have been met in any particular medium. The provision of

proteins in the solid state is complicated by the fact that often they cannot be maintained in finely divided form. In consequence the study of protein requirements was carried out with proteins in solution.

In order to determine the optimum concentration of protein for the mosquito larva, soluble casein was tested at various levels, with best results between 0.5 and 1%. Within this range the omission of glucose from the medium had, if anything, a beneficial effect. Lower concentrations of casein were inadequate to sustain growth (Table 2). The turbidity of soluble casein solutions after autoclaving, increasing as it did during the course of the experiment, made observation of the larvae difficult. We had recourse to sodium caseinate, which gave sparklingly clear media and at 1% concentration produced satisfactory growth and survival rates (Table 3).

Using these results as a standard of comparison, a number of other proteins and related substances were tested and it was clear that proteins known to

Table 2 *The optimum level of protein in the larval medium*

(Medium CM, with changes as indicated)

Concentration of soluble casein (%)	Concentration of glucose (%)	No. of second larval instars	No. of adult mosquitoes	Total time taken (days)	
				Mean	S.D.
0	2	0	—	—	—
0.1	2	0	—	—	—
0.2	0	19	0	—	—
0.2	2	21	2	11.0	—
0.5	0	17	11	11.5 ± 1.14	—
0.5	2	11	7	13.4 ± 1.21	—
1.0	2	33	20	11.0 ± 1.40	—
2.5	2	37	14	10.8 ± 1.32	—

Table 3 *The growth-promoting powers of various proteins*

(No = number in stage of development indicated. The number of first-instar larvae taken was approximately 20 per experiment. Mean, s.d. = mean and standard deviation of time (in days) taken to change from previous stage of development.)

Protein tested (in solution in growth medium CM)	Concen- tration of protein (%)	Larval instars								Adults				
		II			III		IV			Pupae		Total time taken		
		No	Mean,	s d	No	Mean, s d	No	Mean, s d	No	Mean, s d	No	Range	Mean, s d	
Sodium caseinate	1.0	40	2.1±0.67		40	1.5±0.42	39	1.9±0.51	34	3.4±0.65	32	9.5-13.0	10.8±1.09	
Ovalbumin	0.5	31	2.8±0.67		31	2.3±0.87	31	2.0±0.56	24	5.9±1.93	21	11.0-18.0	14.6±2.11	
Gelatin	0.5	10	1.5±0.71		8	1.0±0.83	4	0.60	3	8.7	2	20.0-29.0	24.5	
Gelatin	1.0	19	8.4±5.96		17	7.4±2.65	10	6.0±1.20	3	7.8	3	23.5-28.5	26.2	
Edestin	1.0	20	2.6±0.69		20	2.1±0.45	20	2.4±1.09	18	5.4±1.71	17	10.5-20.5	14.3±1.93	
Ovomucoid	1.0	21	4.6±1.43		20	5.3±2.52	18	6.9±3.50	10	8.8±2.19	7	18.0-33.0	22.8±4.64	
Yeast nucleo- protein	1.0	14	3.3±1.48		12	1.5±0.32	12	1.9±0.30	10	4.1±0.62	10	11.0-16.5	13.1±1.72	
Sericin (soluble)	0.5	0	No growth	—	—	—	—	—	—	—	—	—	—	
Beef peptone	1-2	0	No growth	—	—	—	—	—	—	—	—	—	—	
Tryptic digest of casein	1.0	22	4.1±1.70		22	2.8±0.78	22	2.8±0.69	20	4.1±0.77	20	13.5-18.5	15.6±1.41	

be of inferior nutritive value for animals were equally unsatisfactory for the mosquito larva. Such nutritive inadequacy was revealed by decreased rates of growth and of survival (Table 3).

Casein digests and hydrolysates. With the exception of tryptic digest of casein which, as can be seen from Table 3, produced a good response, other casein digests and hydrolysates proved disappointing. Papain casein digest gave some stimulus to growth but acid hydrolysates were toxic, even after removal of chlorides and supplementation with amino acids. In spite of all efforts to free the hydrolysates from humin and other possible toxic ingredients, they produced no growth.

Amino-acid mixtures. Preliminary studies were carried out with amino acid mixtures used to supplement ovalbumin and gelatin. Details of the composition of these mixtures have been given in Table 1. Eleven amino acids, in conjunction with 1% gelatin solution, produced a striking growth response (Table 4). With media containing amino

acids but no protein, one of the mixtures tested was *B*, consisting of 19 amino acids in a concentration of 0.67%. Here growth was greatly delayed and survival poor. When the proportions were changed and the total concentration raised to 1.06% (mixture *C*) the results were more encouraging. Because of their possible toxic effects, aspartic and glutamic acids were next omitted and the amino acid mixture *D* provided for excellent growth and survival. Mixture *I*, consisting of the same amino acids in different proportions (more emphasis on essential amino acids) was far less effective.

The next step was to omit amino acids which, by analogy with requirements of higher organisms, might be expected to prove dispensable. The series of mixtures *N*, *O*, *P* and *R*, containing 14, 14, 13 and 11 amino acids respectively in concentrations of 0.8-1.1% all gave results decidedly inferior to those obtained with mixture *D*. Mixtures *C* and *D* were also tested at one half of their previous concentrations. Although both media still contained

Table 4 *Growth of mosquito larvae on media containing mixtures of amino-acids*

Protein	Amino acid mixture	No of amino acids in mixture	No of second larval instars	No of adult mosquitoes	Total time taken (days)	
					Range	Mean s.d.
Ovalbumin (0.5%)	X	6	20	5	12.5-28	19.1
Gelatin (1.0%)	X	6	19	12	16.5-23	18.5±1.52
Gelatin (1.0%)	Y	11	21	21	10.5-15.5	12.5±1.05
Gelatin (1.0%)	Y without tyrosine	10	23	19	10.5-15.5	12.3±1.31
	B	19	21	10	18-33	22.6±4.20
	C	19	20	14	10.5-16.5	13.4±1.65
	D	17	19	17	9.5-16	12.6±2.20
	I	17	22	13	16.5-26	22.7±2.83
	N	14	21	16	12-26	15.5±3.15
	O	14	34	16	17-29	23.3±3.67
	P	13	24	20	17-23.5	19.3±2.56
	R	11	24	13	8.5-30.5	23.0±6.91
	C (half strength)	19	23	13	16-29.5	19.1±4.18
	D (half strength)	17	18	14	14-28	21.3±4.10

approximately 0.5% of amino acids, growth and survival were adversely affected

Attempts to eliminate yeast autolysate from media
In order to study the amino acid requirements of the larva it was desirable that the basal medium should be free from such amino acids as might be introduced by the yeast autolysate. Efforts were made to replace the autolysate by pteroylglutamic acid and the lactone of 4-pyridoxic acid which, as reported by us (Golberg & De Meillon, 1947) are capable of producing good growth in sodium caseinate media. Replacement of sodium caseinate by amino acid mixtures such as *D* failed to produce growth, although there was no evidence of toxicity.

Attempts to concentrate the active principles from yeast autolysate by methods previously used for urine (Golberg, De Meillon & Murray, 1947) were successful only as long as caseinate was used in the larval medium, and only in the initial stages of the concentration process.

One further possibility was a reduction in the concentration of yeast autolysate, from what might appear to be the unduly high level of 2%. Since each experiment often lasted for well over a month,

the deterioration of the medium in the course of prolonged incubation made it necessary to have an initially high level of the unstable growth factors.

Amino-acid requirements

With amino-acid mixture *D* as the starting point, each component amino-acid was omitted in turn. For convenience the results have been recorded separately in Tables 5 and 6, according as the amino acids omitted proved to be 'dispensable' or 'indispensable'. The larval response was by no means as clear cut as this division would suggest. In every case the survival rate was smaller and the rate of growth to a greater or lesser extent delayed. In some cases the tests were also carried out using as the basal medium mixture *I*, which contained much smaller proportions of 'dispensable' amino acids. This time the omission of individual 'dispensable' amino-acids made little difference to the larval response.

In contrast to the somewhat ambiguous results with 'dispensable' amino acids, it was quite clear that the following were quite indispensable: glycine, L-leucine, DL-isoleucine, L-histidine, L-arginine, L-

Table 5 *The effects on growth of mosquito larvae of the omission of a single amino acid*

Amino acid mixture	Amino acid omitted	No of second larval instars	No of adult mosquitoes	Total time taken (days)	
				Range	Mean s.d.
<i>D</i>	DL-Alanine	23	13	15.5-27.5	19.5±3.61
<i>I</i>	DL-Alanine	19	9	19.5-31.5	25.6±3.42
<i>D</i>	DL-Valine	21	9	13.5-25	18.7±3.0
<i>I</i>	DL-Valine	21	15	16-25	19.6±2.34
<i>D</i>	DL-Proline	17	12	14-21	16.5±1.89
<i>I</i>	DL-Proline	18	10	18-28.5	24.5±2.07
<i>D</i>	L-Hydroxyproline	17	7	11-22	13.9±3.52
<i>I</i>	L-Hydroxyproline	22	14	19-30.5	25.7±3.13
<i>D</i>	DL-Serine	21	9	15-18	15.7±1.25
<i>I</i>	DL-Serine	20	12	18.5-31	26.0±3.39
<i>D</i>	L-Cystine	34	15	14-27.5	19.7±3.03
<i>D</i>	L-Tyrosine	20	11	16.5-24.5	20.5±2.74
<i>D</i>	DL-Phenylalanine	20	10	11-19.5	13.5±2.52
<i>N</i>	DL-Phenylalanine	20	17	12.5-28.5	19.8±4.06

Table 6 *The effects on growth of mosquito larvae of the omission of amino acids**

Amino acid mixture used	Amino acids omitted	Larval instars						Pupae	
		II		III		IV		No	Mean
		No	Mean, s.d.	No	Mean, s.d.	No	Mean, s.d.		
<i>O</i>	Glycine	2	5.0	0	—	—	—	—	—
<i>D</i>	Glycine	22	7.3±1.67	22	10.5±2.12	6	7.6±1.94	0	—
<i>O</i>	L-Leucine	16	9.1±2.74	3	10.3	0	—	—	—
<i>D</i>	DL-Isoleucine	19	4.4±1.17	18	4.0±0.91	5	7.1±1.02	0	—
<i>O</i>	L-Histidine	0	—	—	—	—	—	—	—
<i>O</i>	L-Arginine	0	—	—	—	—	—	—	—
<i>O</i>	L-Lysine	18	9.8±3.07	12	8.7±1.89	0	—	—	—
<i>D</i>	L-Tryptophan	14	7.0±2.77	14	8.0±3.41	4	7.5±1.66	1	11.0
<i>O</i>	DL-Threonine	17	8.6±2.91	5	6.1±2.20	0	—	—	—
<i>D</i>	DL-Phenylalanine and L-tyrosine	0	—	—	—	—	—	—	—
<i>D</i>	DL-Methionine	3	11.3	0	—	—	—	—	—

* For explanation of headings see Table 3

lysine, L tryptophan, DL threonine, DL-methionine. The case of DL phenylalanine and L-tyrosine will be considered below.

The sulphur-containing amino-acids In the experiments just described a curious phenomenon was observed in media from which cystine was omitted. Despite the presence of large amounts of DL methionine in each medium, the great majority of the adults formed from the pupae were found dead in the medium, with only the upper half of the body projecting from the pupal case. Examination of the adults failed to reveal any obvious malformations. In addition, the growth and survival rates were reduced and could not be improved by the addition of extra amounts of methionine or DL-homocystine or glutathione. However, in the case of glutathione all the adults emerged, apparently quite normally (Table 7).

Glycine and arginine Attempts to replace glycine by other compounds were partially successful in the case of glutathione but not when DL serine or creatine were used. Similarly, L arginine could be replaced to some extent by DL citrulline, to a slight

extent by L ornithine, but not at all by creatine (Table 8).

Phenylalanine and tyrosine In the experiments recorded in Table 5, neither L-tyrosine nor DL phenylalanine alone could be shown to be essential, although the omission of both prevented all growth (Table 6).

Effects on cuticular pigmentation

The mosquito larva possesses chitinous plates on the head, siphon and saddle, which become more extensive as the larva approaches pupation. It is these circumscribed areas which are darkly pigmented from the second to the fourth instar, in contrast to the more diffuse pigmentation of the pupa. In most media the shade of pigmentation of the larvae varied from deep brown to jet black, but on one occasion it was noted that larvae grown on a papain liver digest medium were extraordinarily pale (Golberg, De Meillon & Lavoipierre, 1945). At the time this observation could not be repeated.

In the present series of experiments pale, unpigmented larvae were first observed in media con-

Table 7 *Effects of sulphur-containing amino-acids on the growth of mosquito larvae*

(Amino acid mixture D used in basal medium.)

Additions to medium	No of second larval instars	No of adult mosquitoes	Total time taken (days)		
			Range	Mean	s.d.
(a) Methionine omitted from amino acid mixture D					
None	3	0	—	—	—
L-Cystine (0.4 g/l.)	13	2	17-22	19.5	—
L-Cystine (0.4 g/l.) + choline (0.8 g/l.)	14	1	—	19	—
DL-Homocystine (0.6 g/l.) + choline (1 g/l.)	20	2	—	24	—
DL-Homocystine (1.0 g/l.) + choline (1 g/l.)	8	0	—	—	—
DL Homocystine (1.0 g/l.) + choline (1 g/l.)	0	—	—	—	—
(b) Cystine omitted from amino acid mixture D					
None	34	15*	14-27.5	19.7±3.03	—
DL-Methionine (0.4 g/l.)	15	4†	17.5-24	20.0±2.47	—
DL Homocystine (0.06 g/l.)	21	12‡	15.5-33.5	21.5±5.11	—
Glutathione (0.2 g/l.)	18	9	17-25.5	21.2±3.15	—

Numbers of adult mosquitoes which died while emerging * = 11, † = 4, ‡ = 10

Table 8 *Effect of arginine and glycine on the growth of mosquito larvae*

Compound added	No of second larval instars	No of adult mosquitoes	Total time taken (days)		
			Range	Mean	s.d.
(a) L-Arginine omitted from amino-acid mixture O					
—	0	—	—	—	—
Citrulline (0.6 g/l.)	21	11	16-30.5	21.4 ± 4.57	—
Ornithine (0.6 g/l.)	22	1	—	29.5	—
Creatine (0.8 g/l.)	0	—	—	—	—
(b) Glycine omitted from amino acid mixture O					
—	2	0	—	—	—
Serine (0.2 g/l.)	11	0	—	—	—
Creatine (1.0 g/l.)	4	0	—	—	—
Glutathione (1.4 g/l.)	17	5	17-26	23.7 ± 3.52	—

taining gelatin as the sole source of protein. The ghost-like appearance of the larvae was due to lack of pigment in the chitinous areas described. Supplementation with amino acids, including phenylalanine and/or tyrosine restored the pigment. An interesting feature of these experiments was the fact that the pale larvae gave rise to pupae which were partially pigmented and, as far as could be ascertained, the adults possessed their full characteristic pigmentation. It was also noted that lack of pigmentation had little, if any, definitely adverse effect on growth or survival (Table 5).

In the case of amino acid mixture *D* the omission of tyrosine produced pale larvae and almost completely unpigmented pupae, but the omission of phenylalanine appeared not to affect pigmentation. This apparent paradox was resolved by increasing the level of phenylalanine in the tyrosine free medium. At a level of 1.0 g DL phenylalanine/l of medium the larvae were pale up to the third instar, but the fourth instar larvae were dark and the pupae appeared normal. At a level of 1.4 g of DL phenylalanine pigmentation was normal throughout.

Intermediate degrees of pigmentation. Observation of fourth-stage larval pelts or, where these were not available, of early instar larvae, indicated the existence of the following intermediate degrees of pigmentation between the colourless larva and the dark normal shade.

- 0 White, with no pigment visible
- 1 Siphon and saddle clear yellow, no darkening
- 2 Siphon and saddle bright yellow, no darkening
- 3 Slight darkening of siphon and saddle
- 4 Siphon definitely darkened, saddle with a large, clearly marked area dorsally

It was thus a matter of interest to us to assess the shades of pigmentation resulting from the use of various possible precursors of melanin in a basal medium capable of producing colourless larvae but fairly good growth and survival. Some of the compounds tested were too toxic to permit growth to the fourth instar, but the remainder gave rise to varying degrees of pigmentation (Table 9).

Table 9 *Pigmentation induced in mosquito larvae by various compounds*

(Medium contained 0.5% gelatin and amino acid mixture *Y* with tyrosine and phenylalanine omitted. All compounds tested at a concentration of 0.4 g/l.)

Compound tested	Specimens examined	Degree of pigmentation*
DL-Phenylalanine	Fourth stage pelts	1
L-Tyrosine	Fourth stage pelts	4
Tyramine hydrochloride	Fourth stage pelts	1
L-3 Aminotyrosine	Fourth stage pelts	2
L-3 4-Dihydroxyphenylalanine	Third stage larvae	0
DL- α Aminophenylacetic acid	Fourth stage pelts	1
p Aminophenylacetic acid	Fourth stage pelts	2-3
L-3 5 Dihomotyrosine	Third stage larvae	0
Adrenaline	Second stage larvae	0

* The significance of the numbers used is explained in the text, p 384

DISCUSSION

Insects offer a striking contrast to higher animals in that many can dispense with dietary nitrogen during adult life (Uvarov, 1928). The proteins utilized during this time must be drawn from reserves accumulated during larval life. An additional factor to be taken into consideration in many instances is the rapid rate of growth. These factors operating together ensure that the mosquito larva is highly exacting with regard to its protein requirements. Failure to obtain protein prevents growth even to the second instar. Failure to obtain protein of adequate quality may have the same effect or may prolong the time of development far beyond its normal span.

For optimum growth in our media the mosquito larva requires a level of protein as high as 1% and, apparently, equally high concentrations of total amino acids. In earlier experiments (Golberg *et al* 1945) we had found that a very light autoclaved suspension of micro organisms sufficed for excellent growth of the larvae. In fact, it was frequently observed in contaminated media that before the presence of the contaminants was visible to the naked eye it was betrayed by a distinct acceleration of larval growth. The high level of protein and of amino acids found necessary in our experiments may be a consequence of the nature of the media employed or of the fact that the mosquito larva is equipped physiologically to deal with discrete particles rather than solutions.

In this connexion attention should be drawn to the remarkably high levels of amino acid nitrogen observed in the blood of insect larvae and chrysalids (Bishop, Briggs & Ronzoni, 1925; Courtois, 1928; Duval, Porter & Courtois, 1928). Values as high as 4 g amino nitrogen/l have been reported, corresponding to well over 20 g of amino acids/l of haemolymph. Such a consequence of histolytic changes probably obtains in the mosquito larva and would explain the necessity for the high level of dissolved protein or amino acids.

The mosquito larva is exacting in its requirement for 'complete' proteins. In this respect it resembles *Drosophila* (Lafon, 1938), *Blatella* (McCay, 1933) and *Pyrausta* (Bottger, 1942). Lafon & Teissier (1939) have drawn attention to the suitability of yeast proteins for the nutrition of *Tenebrio*, and their remarks apply to many other insects, including the mosquito larva.

Our inability to use casein hydrolysates for the mosquito larva recalls a similar experience reported by van't Hoog (1935) with *Drosophila*. Lafon (1938) and Tatum (1941) met the protein needs of *Drosophila* by means of casein hydrolysates supplemented by cystine and/or tryptophan. On the other hand, Kozhanchikov (1944), Lafon (1938) and Buddington (1941) met with little or no success in their efforts to replace proteins by amino acids in insect diets. Our results with mixtures of amino acids show clearly that it is not only the qualitative but also the quantitative composition of an amino acid mixture which determines its growth stimulating ability. By demonstrating the improvement in the rates of growth and survival which is brought about by the presence of non-essential amino acids in adequate proportions these experiments lay emphasis on the antagonistic and toxic effects of amino acids (Gladstone, 1939, Hutchings & Peterson, 1943, Pelczar & Porter, 1943, Porter & Meyers, 1945).

The suggestion that strepogenin (Woolley, 1946 and earlier references) is required by the mosquito larva would serve to explain the fact that growth on amino acid mixtures is in general inferior to that which results from the use of casein, and that pteroylglutamic acid and the lactone of 4 pyridoxic acid can produce growth with sodium caseinate but not with amino acids. However, proteins such as egg albumin and gelatin, known to contain little strepogenin (Sprince & Woolley, 1945), produced slow growth, and when supplemented with amino-acid mixture Y, gelatin led to growth not greatly inferior to that obtained with casein.

Essential amino-acids In connexion with larval requirements of amino acids the concept of 'essentiality' has a special significance, since provision must be made not only for growth but at the same time for maintenance of the adult. It is interesting to observe, however, how closely the requirements of the larva resemble those of higher animals, in particular those of the chick. Thus glycine and arginine are indispensable amino acids for the chick (Klose, Stokstad & Almquist, 1938, Almquist & Meechi, 1940, Hegsted, Hier, Elvehjem & Hart, 1941), they are also indispensable for the mosquito larva. The chick can utilize citrulline but not ornithine in place of arginine (Klose & Almquist, 1940, Klose *et al.* 1938). The mosquito larva is able to grow and metamorphose when given citrulline, it does utilize ornithine to a small extent, since there

was delayed growth to the fourth instar. (The maximum times recorded were to the second stage, 18.5 days, to the third, 20 days, to the fourth, 15 days, to the pupa, 12 days. Many larvae were still alive when the experiment was discontinued.) There is a striking similarity between these observations and the growth of *Streptococcus haemolyticus* on a medium in which arginine was replaced by ornithine. Gale (1945) reported that with ornithine growth was only one quarter of that with arginine and was also irregular. Although isotope experiments have demonstrated the conversion of L-serine into glycine in the rat and guinea pig (Shemin, 1946), Almquist & Grau (1944) found that the omission or inclusion of serine in chick diets had no effect on their need for glycine. Similarly, the mosquito larva was unable to utilize serine in place of glycine. With glutathione its response was definite but delayed and incomplete. In contrast to its effectiveness in the chick (Almquist, Meechi & Kratzer, 1941, Hegsted *et al.* 1941) creatine proved incapable of replacing glycine in the larval diet.

Little is known concerning the role of glycine in invertebrates. Kutscher & Ackermann (1933) noted the surprising fact that glycine betaine has not been found in insects, although it definitely occurs in Crustacea. They attributed this observation to the rapidity of insect metabolism, which prevents the accumulation of glycine in appreciable quantity and hence the formation of betaine.

Valine Microbiological assay of valine using *Lactobacillus arabinosus* revealed a concentration of 29 mg valine per g of yeast autolysate. It is doubtful whether in such a case the entire response of the test organism was due to valine, but the result accounts for the finding that growth of the mosquito larva is not seriously affected by lack of added valine in the medium. A final decision on the essentiality of valine for the mosquito larva will have to be deferred until the existing difficulties in devising a suitable test medium have been overcome.

Sulphur-containing amino acids That cystine is a dispensable amino acid is generally accepted. So much so, that most investigators of recent years have made no effort to ascertain whether in fact cystine is replaceable in all its functions by methionine. Hegsted (1944) acknowledged this omission in studies on the amino acid requirements of chicks. There exists considerable evidence, especially among micro organisms, that cystine plays an indispensable part in nutrition in certain circumstances. Such observations give added point to the effect of cystine lack on the mosquito larva. The failure of a large proportion of adults to emerge successfully suggests that a deficiency of cystine is possible, despite the high level of methionine in the medium. Moreover, while cystine and homocystine are both capable of replacing methionine to some extent, only gluta-

thione could replace cystine in its ability to promote normal emergence of adults

Phenylalanine and tyrosine Mosquito larvae seem equally capable of utilizing either phenylalanine or tyrosine, a fact which might be explained by the presence of a small quantity of phenylalanine in the yeast autolysate used. This suggestion fails to account for the fact that growth is delayed in the absence of tyrosine but remains unaffected when phenylalanine is omitted from the medium.

The production of unpigmented larvae on media lacking tyrosine or phenylalanine is, to our knowledge, the first demonstration that the absence of these amino acids from the diet can affect pigmentation. It is instructive to consider the quantitative aspect of these experiments. Since all media contained equal amounts of yeast autolysate, this factor remained constant. From the values given by Hodson & Krueger (1946), a 1% solution of sodium caseinate would contain 0.48 g/l phenylalanine and 0.53 g/l tyrosine/l. This medium produces full pigmentation. In an amino acid medium, DL phenylalanine, in a concentration of 1.4 g/l, also produces darkly pigmented larvae, but a level of 1.0 g/l allows pigmentation of the fourth stage larva but not of the earlier instars, 0.6 g/l produced no visible pigmentation. In 0.5% gelatin the amount of phenylalanine present would be 0.13 g/l (calculated from the analysis of Block, Jervis, Bolling & Webb, 1940). An additional 0.4 g/l of L tyrosine is sufficient for the dark pigmentation of fourth instar larvae, but the same amount of DL phenylalanine produces pale yellow pigmentation. Thus both with amino acid mixture D and with gelatin it is clear that L tyrosine is greatly superior to DL phenylalanine in its ability to promote the formation of melanin. It may be that D phenylalanine is not utilizable for melanin formation—in *in vivo* only L 3,4-dihydroxyphenylalanine is acted upon by dopa oxidase (Sumner & Somers, 1943). Taking into account the strong melanin forming powers of *p*-aminophenylacetic acid (Table 9) it seems more probable that DL phenylalanine is utilized for the most part for other, more essential, purposes.

The activity of *p*-aminophenylacetic acid, and even to a small extent of α -aminophenylacetic acid, indicates that compounds of various types must be taken into consideration as precursors of melanin *in vivo*. The findings of Pryor, Russell & Todd (1946) that the phenolic substance responsible for the hardening of the cockroach ootheca is protocatechuic acid, in addition to the results of earlier workers, suggests that a wide range of compounds may possibly play a part in the darkening of the insect cuticle.

Serra (1946) has shown that melanins extracted from hairs of different hues contain varying proportions of melanoid pigment and protein. Ac-

cording to his analyses, a combination of 60% melanoid pigment and 40% protein leads to a black melanoprotein, but 30% pigment and 70% protein constitutes a yellow melanoprotein. The colours observed in the head, siphon and saddle of the mosquito larva are therefore indicative of the relative proportions of melanoid pigment formed under the experimental conditions employed. From our results it becomes clear that the production of melanin is a process depending directly on the level of dietary phenylalanine and tyrosine—or, more correctly, on the excess over the quantities required for growth and protein synthesis. In consequence of this fact, varying degrees of melanic pigmentation can be produced at will and absence of pigmentation during the early instars, when all available phenylalanine is presumably required for protein synthesis, can be followed by the production of some melanin when the larva is full-grown.

Our observation that in the mosquito larva pigmentation has little connexion with growth underlines the statement made by Wigglesworth (1942) that 'the pigmented constituents of insects, in some cases are, perhaps, substances of physiological importance, but the majority seem to be merely by-products of metabolism'. Since tanning by means of a melanin precursor plays an essential part in the hardening of the insect cuticle (Pryor, 1940; Trim, 1941; Hurst, 1940, 1945) it is possible that in unpigmented larvae the cuticle remains soft. The physiological implications cannot be discussed here. There is obviously a wide scope for the use of the nutritional approach to the study of melanin formation *in vivo* for the elucidation of the many problems as yet unsolved in this field.

SUMMARY

1 In a medium complete in other respects, but lacking protein the mosquito larva does not grow to the second instar.

2 After yeast, casein is the most suitable protein, but incomplete proteins, on supplementation with amino acids, prove adequate for growth and survival to the adult stage.

3 Good rates of growth and survival could be produced by using mixtures of amino acids, but variation in their relative proportions or reduction in the number of amino acids had adverse effects.

4 By omitting single amino acids from a mixture, it was possible to establish that the following are essential for the mosquito larva: glycine, L leucine, DL isoleucine, L-histidine, L-arginine, L-lysine, L-tryptophan, DL-threonine, DL phenylalanine and DL methionine. The status of DL valine could not be determined while using yeast autolysate in the medium.

5 There was evidence that the omission of L-cystine resulted in a high proportion of adult mosquitoes failing to emerge

6 According to the level of phenylalanine or tyrosine in the medium various shades of pigmentation could be produced in the mosquito larvae. The adults emerging even from wholly unpigmented larvae were normally pigmented and there appeared

to be little relation between pigmentation and growth or survival. A number of compounds were found capable of producing intermediate degrees of pigmentation in the mosquito larva

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The Chemistry of Connective Tissues

1 THE STATE OF COMBINATION OF CHONDROITIN SULPHATE IN CARTILAGE

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In recent years evidence has been presented by several groups of workers that chondroitin sulphate exists in tissues such as cartilage in a very highly polymerized condition. The early workers in the field regarded chondroitin sulphate as an oligo-saccharide of quite low molecular weight, and Levene (1925) formulated the substance as a non-reducing tetrasaccharide composed of two residues of glucuronic acid and two residues of a sulphate of

N-acetylglucosamine. Perhaps the main reason for the failure of the early workers to recognize the highly polymerized character of the polysaccharide lay in the fact that strongly alkaline reagents were invariably used to effect the initial extraction from the tissue, and it has since been shown that the polysaccharide is rapidly degraded in the presence of strong alkali, particularly at temperatures above 0°.

The quantitative extraction of chondroitin sulphate from cartilage remains a matter of considerable difficulty, and dilute NaOH is still frequently employed. This method was investigated by Jorpes (1929), who showed that a concentration of NaOH as high as 0.5N was necessary to extract the acid polysaccharide from cartilage at 0°. Meyer & Smyth (1937) suggested the use of strong solutions of calcium, strontium or barium chlorides, and obtained good yields of chondroitin sulphate by the extraction of dried cartilage powder with 10% (w/v) CaCl_2 solution at neutral reaction.

Blix & Snellman (1945) used CaCl_2 extraction and were careful to avoid acids or alkalis during the subsequent separation of the polysaccharide from the protein moiety of the extracted mucoid. Prepared under careful conditions, the chondroitin sulphate was shown to have a molecular weight of the order of 260,000 and a long chain structure was suggested. In dilute alkali, the molecule was depolymerized at room temperature to a rather definite point, at which the products formed were still of colloidal size, although giving little double refraction of flow. The molecular weight of chondroitin sulphate extracted from cartilage by dilute alkali was estimated to be of the order of 40,000–50,000.

Meyer & Odier (1946) prepared chondroitin sulphate by extraction of hog nasal cartilage in 0.5N-NaOH at 5°. Their preparation was probably similar to the alkali degraded polysaccharide of Blix & Snellman (1945), and from viscosity measurements calculated on the basis of an unbranched chain its molecular weight was estimated at 10,000–15,000. The reducing power of the polysaccharide towards NaIO at pH 10.5 and towards HIO_4 at pH 4.2–4.4 was investigated, and from the results obtained it was concluded that the residues of glucuronic acid and *N*-acetylglucosamine 6 sulphate are arranged to form an unbranched chain molecule. This conclusion is in opposition to the earlier view of Bray, Gregory & Stacey (1944) who methylated a degraded chondroitin that had been obtained by acid hydrolysis of a chondroitin sulphate prepared with alkali. From isolation of the products of hydrolysis of the methylated oligosaccharide it was concluded that chondroitin sulphate possesses a structure of the branched chain type.

The chondroitin sulphate of hyaline cartilage is associated with the semi-transparent, apparently structureless, ground substance that constitutes the bulk of the tissue and gives the cartilage its characteristic physical properties. The cartilage cells occupy cavities in this ground substance and appear to be isolated from one another. In spite of its apparent homogeneity, histological studies show that the intercellular ground substance is not amorphous since after digestion with trypsin or by use of the various silver impregnation methods, collagenous fibrils are discernible which form a dense felt-work running in all directions or gathered together in oriented bundles. From its histochemical reactions the collagen of the ground substance of cartilage is considered to be similar to that occurring in loose connective tissue. When cartilage is extracted with alkalis or with solutions of inorganic salts, part of the material passes into solution and constitutes the so-called 'chondromucoid', the residue is mainly collagen but retains more or less mucoid according to the efficiency of the agent used for extraction. The chondromucoid contains protein and gives precipitates on addition of acetic acid which usually take the form of clear firm gels.

The nature of the protein moiety of chondromucoid and

its relation to the chondroitin sulphate and the collagen structures of the tissue itself have received relatively little attention. Bungenberg de Jong & Dekker (1935) studied the interaction of chondroitin sulphate with proteins and showed the presence of complexes which were regarded as 'coacervates'. Meyer, Palmer & Smyth (1937), on the other hand, looked upon the interaction as a salt formation and showed that in the presence of acetic acid true salts are formed in stoichiometric proportions by the union of the basic groups of the protein with the acid groups of the polysaccharide. It was shown that in the presence of 2% (w/v) acetic acid, the amount of chondroitin sulphate combined by a protein corresponded to the amount of basic amino acids contained in the protein, and further, the chondroitin sulphate binding power of the protein was in agreement with the acid binding power found by studies of dye protein complexes. In the salts studied the chondroitin sulphate was found to react with the protein as a dibasic acid, both the sulphuric acid group and the carboxyl group of the uronic acid residue take part in salt formation. Meyer *et al.* (1937) were of the opinion that chondroitin sulphate occurs in nature in salt-like complexes which are present as structurally organized elements of sheet or fibre form, and pointed out that analysis of ground cartilage showed the ratio of hexosamine N to total N to be 0.046, a figure that compares well with the corresponding ratio 0.056 for the gelatin salt of chondroitin sulphate. This close correlation led to the view that the major portion of the cartilage is a protein salt of chondroitin sulphate.

This suggestion is not borne out by the experiments of Blix (1940), who submitted an aqueous extract of cartilage (nasal septum of ox) to electrophoresis in the apparatus of Tiselius (1937) at hydrogen ion concentrations within the physiological range. Two components only appeared, the faster being almost pure chondroitin sulphate while the slower was a protein containing only 0.02% hexosamine. No evidence of combination at a higher pH than the isoelectric point of the protein was reported, and the result appears to support the earlier view of Möerner (1889) that in cartilage the acid polysaccharide is at least partly present as alkali salt and is, therefore, not fully combined with protein. It should be pointed out, however, that only a very small proportion of the chondroitin sulphate is extractable from fresh cartilage by water under the conditions used by Blix, and the extracted mucoid may not be in the same form as the material remaining in the tissue.

The intention of the work described here was to make a preliminary study of the chemical structure of the intact tissue, and in embarking on this problem it was considered that a possible line of approach would lie in the application of a process of successive mild degradation of one or other of the components of the tissue, followed by an examination of the breakdown products that resulted. The process of degradation of collagen fibres as they occur in tendon or hide has already received much attention, particularly by workers interested in the mechanical properties of leather, and for this reason the first approach has been a study of the liberation of chondromucoid under conditions that would be expected to give rise to thermal degradation of the collagen structures present in the tissue.

The work of Lloyd & Garrod (1946) has shown that collagen fibres of tendons (from the tails of rats) rapidly contract to about one third of their original length when heated in water to 60–70°, and that in the contracted state the fibres show a new property of rubber like elastic extension. Lloyd and her colleagues found that formamide, strong solutions of the phenols and anhydrous formic acid are very effective in reducing the temperature at which thermal contraction takes place. Strongly acid (pH 2) or alkaline (pH 12) solutions and concentrated solutions of salts are also effective. Among the salts the effect of the lyotropic series is evident and, in addition, Ca^{++} is stated to be particularly effective in causing shortening in length at low temperatures.

Since published data on the thermal contraction of collagen fibres were insufficient to afford a direct comparison with the methods devised for the extraction of cartilage, a number of experiments were carried out during the course of this work to determine the effect on rat tail tendon of the temperature and concentration conditions used in the extraction experiments, and these are reported in the experimental section.

It is probable that the chondroitin sulphate liberated under the rather mild conditions that give rise to thermal shrinking of collagen fibres remains in a condition closely approaching its state in the tissue itself and, with this in mind, it was considered that a study of the physical and chemical properties of the extracted mucoid would be of value. Evidence of complex formation between the undegraded polysaccharide and the protein of the mucoid under conditions of pH and salt concentration in the physiological range is of particular interest, and for this reason a preliminary electrophoretic study of the material extracted by the new method has been undertaken.

EXPERIMENTAL

Analytical methods

A modification (Lugg, 1938) of the peroxide fusion method was used for the estimation of total S. Ester bound S was determined as SO_4 S after hydrolysis with 5N HCl for 20 hr at 100°, using the gravimetric procedure described by Lugg (1938). The micro Kjeldahl method was used for the determination of N. Moisture was determined by drying to constant weight over P_2O_5 at 80° under reduced pressure, and in all cases ash was weighed as sulphate.

All analyses quoted in the text are corrected for ash and water unless it is otherwise stated.

Preparation of the cartilage powder

Fresh cartilage from septum nasi of cattle was freed from adhering connective tissue and finely chopped with a scalpel. The pieces were introduced at once into acetone, and after standing for some time in two or three changes of solvent they were dried in air at 70° and ground in a hammer mill through a sieve with circular apertures 0.56 mm in diameter.

Extraction of cartilage powder with water at room temperature

Although little mucoid could be extracted from fresh cartilage by water at room temperature, even after the tissue had been finely minced, a considerable proportion of the mucoid became extractable after the tissue had been dehydrated in acetone and ground to a powder. Portions of the powdered cartilage (N, 12.4, ester S, 1.2, P, 0.06, ash, 5.6, moisture, 5.6%) were extracted with distilled water by shaking gently for 2–3 hr at room temperature. One extraction was sufficient to remove the bulk of the extractable material, but in order to exhaust the residue, two further similar treatments were given. The chondromucoid was precipitated from the aqueous solution by addition of ethanol (3 vol) after adding a few ml of saturated sodium acetate solution to bring the concentration of the salt to about 0.5% (w/v). The precipitate was washed first with 80% (v/v) ethanol and then thoroughly dehydrated with anhydrous ethanol before drying. Prepared in this way the mucoid was a fine white powder having N, 9.2–11.5, ester S, 3.2–3.3, P, 0.04%, and dissolved in water to give a clear viscous solution. Results obtained from a number of experiments showed that 30–35% of the acid-hydrolyzable S present in the original tissue was extractable by distilled water at 18–23°.

Effect of a short heat treatment on the extraction of cartilage powder

Cartilage powder that had been thoroughly extracted with water at room temperature yielded a further amount of chondromucoid after a short heat treatment in water at 60–80°. Portions of the water-extracted powder (1.55 g) yielded 0.273 g of chondromucoid after heating with distilled water at 80° for 10 min, followed by shaking gently for 2 hr at room temperature. In a subsequent series of experiments it was found that a preparation of cartilage powder that had already yielded 35% of its content of ester S by extraction with cold water yielded a further 30–35% of the ester S originally present in the tissue, on treatment with distilled water at 60° for 30 min. The chondromucoid extracted after heat treatment was similar in composition to that extracted by cold water having N, 10.7–12.4, ester S, 3.1–3.7%. The material remaining insoluble still contained 33–36% of its original content of ester S, having N, 14.7–15.0, ester S, 0.6–0.63%.

Extraction with calcium chloride solution

Extraction with a 10% (w/v) aqueous solution of CaCl_2 was found to be more effective after an initial short period of gentle heating. A sample of cartilage

powder that had already been exhaustively extracted with cold water was warmed to 45° for 30 min with 10 % (w/v) CaCl_2 solution (pH 7.49), followed by cooling to room temperature and shaking for 3 hr. The extracted chondromucoid had N, 8.2, ester S, 3.8 % and represented 28 % of the ester S originally present in the fresh cartilage. The substance after recovery from the CaCl_2 solution was not entirely soluble in distilled water or in dilute salt solutions, and gave a small amount of gelatinous residue. After removal of insoluble material by centrifugation, the aqueous solution was clear and viscous. Since extraction with neutral CaCl_2 solution under these conditions was incomplete, the extraction was repeated with a solution of CaCl_2 (10 % w/v) that had been adjusted to pH 11 by addition of $\text{Ca}(\text{OH})_2$ followed by filtration. The mixture was heated to 37° for 30 min, and in this case a total extraction of 90 % of the ester S was obtained, but the product (N, 11.7, ester S, 3.6 %) did not give highly viscous aqueous solutions and appeared to be more readily soluble in water than the mucoid extracted at neutrality. The residue from the cartilage after a further extraction with the alkaline CaCl_2 reagent had N, 16.5, ester S, 0.15, total S, 0.56 %, and since all but a small residue (6.3 %) was converted to gelatin on heating with water at 120°, the substance was considered to be almost wholly collagen.

Extraction with aqueous solutions of formamide

Freshly prepared solutions of anhydrous formamide (m.p. -2°) were used for these experiments. Neither 10 % (v/v) nor 30 % (v/v) solutions were effective in extracting further mucoid from water extracted cartilage powder at temperatures up to 37°. However, on warming to 45° for 30 min a further 30 % of the ester S originally present in the tissue was extracted by 30 % (v/v) formamide solution, and the product after recovery by precipitation with ethanol (N, 10.0, ester S, 3.3 %) was similar in analytical composition to the product of extraction with hot water or calcium chloride solutions. The mucoid extracted with 30 % (v/v) formamide, redissolved in water to give clear viscous solutions.

Thermal contraction of tendons from the tail of the rat

The procedure adopted was a modification of that of Lloyd & Garrod (1946). The tendons were carefully extracted from rat tails that had been stored for 2 weeks at 0°, and any tendon that had been stretched or damaged in the process of removing it from the tail was rejected. To both ends of each tendon a small lead shot was attached by inserting the end of the tendon into a deep cut in the shot, made by a razor blade, and then gently closing the cleft by means of a pair of forceps.

Table 1 *Thermal contraction of rat tail tendon*

(The length of the tendon at the time indicated is expressed as percentage of the initial length.)

Temp	2 min	5 min	10 min	20 min	60 min
		Solvent distilled water			
55°	100	100	100	100	100
60	49	64	95	97	100
65	47	93	100	103	110
70	33	104	108	112	116
		Solvent 10 % (w/v) CaCl_2 (pH 6.05)			
37°	100	100	100	98	92
45	—	60	53	90	120
50	43	68	88	100	106
		Solvent 10 % (w/v) CaCl_2 (pH 11.2)			
40°	—	86	27	30	116
45	—	27	27	44	111
50	23	23	36	84	118
		Solvent anhydrous formamide			
35°	—	95	67	67	95
40	—	67	69	100	102
45	—	61	100	110	112
		Solvent 30 % (v/v) aqueous formamide			
30°	100	100	100	97	35
40	67	76	95	102	106
45	57	57	66	74	77
		Solvent 10 % (v/v) aqueous formamide			
40°	—	87	27	30	110
45	—	27	27	44	87
50	23	23	36	84	118

The tendon was suspended in a narrow graduated cylinder which contained the solvent, the upper lead shot being gripped in a clip while the lower one hung free. The weight of the lower shot was chosen to be just sufficient to sink the tendon and hold it at full length. The graduations on the cylinder were used as an arbitrary unit for measuring the changes in length of the tendon, and for temperature control the cylinder was placed in a water bath fitted with a stirrer.

The results of a number of experiments are given in Table 1, which shows the changes in length with time at various temperatures and in various solvents. In all experiments, little change in length occurred below a certain limiting temperature, above this temperature, however, rapid contraction took place and this was followed by a slow increase in length so that the final length of the tendon was often greater than the initial value. At this stage the tendon had lost much of its tensile strength and had become partially gelatinous. In the table, only those experiments carried out near the limiting temperature are reported. It should be noted that there was some variation in the behaviour of individual tendons, even among those extracted from the same rat's tail, but the values recorded are typical of the results obtained from many experiments.

Correlation of the conditions resulting in extraction of chondromucoid with those giving rise to thermal contraction in collagen fibres

In Table 2 the results of a number of extraction experiments are given. The sample of cartilage powder used had not been previously extracted with water at room temperature so that the yields given (expressed as percentage ester S extracted from the original tissue) are total yields. It seems clear that a short period of heating materially assists the release of chondromucoid from the remaining con-

stituents of the tissue, either in aqueous solution or in solutions of formamide or salts. Further, the action of CaCl_2 or formamide solutions is not due entirely to an enhanced solubility of the chondromucoid in these reagents, since the substance is readily soluble in cold water, and can in fact be extracted in good yield by distilled water alone after a short heat treatment. Solutions of the active reagents have, however, a marked effect in reducing the temperature of the heat treatment necessary to effect extraction. Comparison of Table 2 with Table 1 shows the marked correlation between the reaction conditions necessary to extract chondromucoid in good yield with those giving rise to thermal contraction of free collagen fibres. It should be pointed out, however, that, except in the experiments with CaCl_2 solution at pH 11, complete extraction was not obtained, and in this one case there is reason to suppose that the mucoid itself suffered degradation since the product gave solutions that were considerably less viscous than those obtained from chondromucoid extracted with less alkaline media. Although there were considerable variations in the N and S contents of the extracted mucoids in individual experiments, the figures given in Table 2, which show the range obtained over a number of duplicate experiments, indicate little systematic variation and show that the product extracted under the varying conditions had substantially the same analytical composition.

Isolation of the protein component

Investigations on mucopolysaccharides of bacterial origin have shown that the solubility of the polysaccharide moiety of the complexes in strong solutions of phenol is in some cases less than that of the protein (cf Morgan & Partridge, 1941). In the case of chondromucoid it was found that at 37° considerable amounts of protein may be extracted from the

Table 2 *Effect of heat treatment on the extraction of cartilage powder in various solvents*

Treatment	Ester S extracted (%)	Chondromucoid	
		Ester S (%)	N (%)
Water at 20°	30-35	3.15-3.3	9.2-11.5
Water at 60°, 30 min	58	3.1	12.4
Water at 80°, 10 min	65-70	3.5-3.7	10.7-11.0
CaCl_2 (pH 7.5) 45°, 30 min	60	3.5-3.8	8.2-9.7
CaCl_2 (pH 7.5) 55°, 30 min	65	3.5-3.6	9.3
CaCl_2 (pH 11) 37°, 30 min	95-98	3.6-3.8	11.7
Formamide 30% (v/v) 37°, 30 min	44	—	—
Formamide 30% (v/v) 45°, 30 min	63	3.3	10.0

dry powder by 90% (w/w) aqueous phenol. The mucoid (2 g, N, 9.41, ester S, 2.33, ash, 9.3, moisture, 6.5%) was allowed to stand at 37° with 90% (w/w) phenol solution (150 ml) for 24 hr, the mixture being occasionally stirred. The residue was removed by centrifugation and the clear solution treated with ethanol (3 vol). The heavy precipitate, after collecting and washing with ethanol in the centrifuge, was dissolved in water at 60° and again precipitated with ethanol. After washing with ethanol and ether the substance was dried at room temperature over P_2O_5 .

Prepared in this way, the substance had N, 17.53, ester S, 0.13, total S, 0.44% and was thus essentially protein in character. The residue from phenol extraction had N, 7.96% (uncorrected for ash and water) and was extracted with further quantities of 90% phenol solution. The yield of protein from the first extract was 0.2 g, but from successive extracts the amount of protein yielded diminished rapidly and after four extractions the N content of the residue remained constant at 7.26% (uncorrected). It was clear, therefore, that although part of the protein contained in the 'undegraded' mucoid was freely extracted by phenol solution, a further part of it was not available for extraction, and in subsequent experiments it was not found possible to reduce the N content of the mucoid below about 7% by repeated extraction with phenol. The protein was not soluble in cold water or cold dilute alkalis or acids, but dissolved readily in water at 50–60° giving clear viscous solutions. On cooling, the solutions set to a firm gel.

Viscosity of the extracts

The dry preparations of chondromucoid were usually not completely soluble in water or buffer solutions, and solubility slowly decreased on storage in the dry state. Solutions of the chondromucoid, on the other hand, were fairly stable, but it was observed from time to time that the viscous solutions slowly became more opalescent on standing at 0° and sometimes gave rise to a fine precipitate.

These effects caused difficulty in making up solutions of the mucoid for viscosity determinations or for electrophoresis experiments and for this reason fractionation with ethanol was eliminated and the extracts obtained in solutions of electrolytes were first dialyzed against distilled water for 48 hr followed by dialysis against the appropriate buffer solution. Concentration was then measured refractometrically by making use of a value for the refractive increment of the mucoid in the buffer solution. Since the composition of the mucoid was rather variable, particularly as regards ash and N content, there was some uncertainty about the value of the refractive increment to be adopted, but from a number of experimental determinations on mucoid

extracted from the cartilage powder in phosphate NaCl buffer of pH 6.90 at 60° an average value of 1.60×10^{-3} (1% (w/v) at 25.2°) was adopted for all the refractometric estimations.

Viscosity determinations were carried out at 25.4° in an Ostwald viscosimeter having a capillary length of about 9 cm and a flow time for water of 31.2 sec. As would be expected the relative viscosity (η_r) of the mucoid in salt solution was much lower than that given by the same preparation in distilled water, for instance, mucoid that had been extracted by distilled water at 60° gave (after dialysis against changes of distilled water) a value for η_r in water of 3.16 for

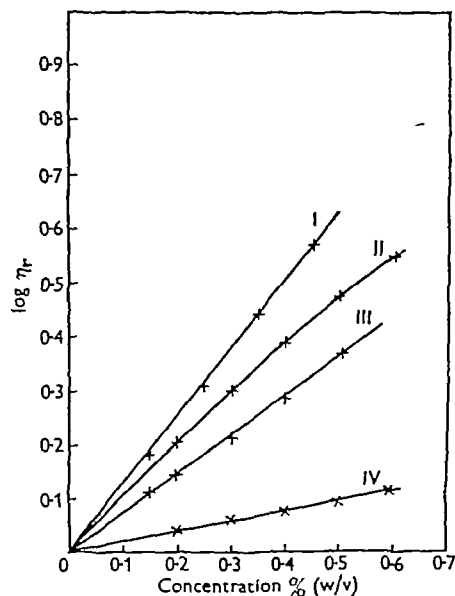


Fig 1 Log η_r for extracts of cartilage, viscosity determinations carried out in phosphate NaCl buffer of pH 6.90, I, μ , phosphate, 0.02, μ , NaCl, 0.20, H₂O at 70°, II, CaCl₂ (pH 7.4) at 55°, III, Phosphate NaCl at 60°, IV, CaCl₂ (pH 11.0) at 37°

a 0.25% solution, while after dialysis against phosphate-NaCl buffer of pH 6.97 (μ , phosphate, 0.02, μ , NaCl, 0.18) the solution had η_r 1.53 at the same concentration. The viscosity of the mucoid, both in water and salt solutions, showed variations from preparation to preparation even under carefully controlled conditions of extraction, but the values for the viscosity of individual preparations at different concentrations lay on smooth curves. The values for log η_r for different dilutions of most of the preparations could be fitted by straight lines, and Fig 1 shows log η_r plotted against concentration for a number of the preparations.

If the equation of the log η_r concentration curve is taken as

$$\log \eta_r = Kc,$$

where K is a constant and c is the concentration of the solute, then, at infinite dilution (provided natural logs are used), $l =$ the 'intrinsic viscosity' $[\eta]$, where

$$[\eta] = \lim_{c \rightarrow 0} \frac{(\eta_r - 1)}{c} \quad (\text{Kraemer \& Lansing, 1935})$$

According to Staudinger (1940) the value of intrinsic viscosity is proportional to particle weight even in the case of heteropolar substances, provided that the particles are linear macromolecules and charge effects are suppressed by carrying out the viscosity determinations in solutions containing a sufficient concentration of electrolyte. However, the solutions of mucoid contain more than one molecular species and, as will appear later, in dilute solutions containing electrolytes chondromucoid may be looked upon as a complex in equilibrium with its dissociation products. Under these conditions it is probable that the significance of $[\eta]$ as a measure of particle length is qualitative only, but since $[\eta]$ for the extracts made under alkaline conditions is of a lower order compared with the values given by the mucoid extracted at neutral reaction, it is clear that in the former case considerable degradation has occurred.

It is implicit in the theory of Staudinger that the effect of molecular association on viscosity depends upon the relative position of the molecules associating together. If compact bundles are formed, the viscosity is supposed not to be affected, while, if two similar molecules associate end to end, the value for $[\eta]$ is twice as great as in the absence of association. The linearity of the $\log \eta_r$ curves is therefore of especial interest since it indicates that, in the presence of electrolytes, the degree of end to end association is not significantly increased with increasing concentration.

Effect of heat treatment on the viscosity of chondromucoid

An extract of chondromucoid was prepared in phosphate NaCl buffer of pH 6.9 (μ , phosphate, 0.02, μ , NaCl, 0.18) and was dialyzed against several changes of the same buffer. Samples of the solution (10 ml, c 0.481% (w/v)) were sealed in glass tubes and heated for varying periods of time, one series being heated at 100° and another at 80°. At both temperatures the viscosity of the solutions fell very rapidly in the first 5 min. of heating, the initial rapid fall being followed by a further slow reduction in viscosity over a period of some hours. The initial viscosity of the solution was η_r 3.12 at 25.4° and after heating 5 min. at 80° the value fell to η_r 1.69, after which, on heating a further 10 hr. at 80°, the value obtained was η_r 1.35. Since, as will appear later, the solution of mucoid contained free gelatin, the slow

continuous fall in viscosity which followed the first dramatic change may have been due to the degradation of this protein (cf. Ames, 1947).

Electrophoresis experiments

The experiments were carried out at 3.2° in the apparatus of Tiselius (1937) using the optical arrangement of Philpot as modified by Svensson (1930, 1946). In order to suppress boundary anomalies and to reduce the effects of viscosity the experiments were carried out with solutions containing high concentrations of salt and low concentrations of colloid. Phosphate-NaCl, glycine NaCl and acetate NaCl buffers were used, the solutions having ionic strength 0.02 with respect to the buffer ions and ionic strength 0.18 with respect to NaCl. The preparations of mucoid and protein were dialyzed against the appropriate buffer for 3-4 days until equilibrium was reached, the concentration of the colloid was then measured refractometrically and the solutions were adjusted to 0.25-0.5% (w/v) by dilution with the buffer. The conductivities of the buffer and mucoid solution were measured at 0° and the mean of the two values was used in conjunction with the milli-ampere readings in order to determine a mean value for the potential gradient at the boundaries. Since conductivity was measured at 0° the values for mobility given in the tables refer to 0° rather than to the temperature at which the experiment was carried out. The pH of the buffer solutions was measured by means of a hydrogen electrode at 0°.

Electrophoresis of mucoid solutions The results of a series of electrophoresis experiments carried out on the same batch of cartilage extract are given in Table 3. The extract was made at 60° in phosphate NaCl buffer of pH 6.9 following the procedure already given, and, for the individual mobility determinations, samples of the extract were dialyzed against buffers of the desired pH.

Fig. 2(a) shows the electrophoretic pattern obtained at pH 8.5 in phosphate NaCl buffer (μ , phosphate, 0.02, μ , NaCl, 0.18). Over a range of pH values from 5.9 to 9.0 the patterns given were essentially similar. In the positive limb of the cell three boundaries were invariably present, and these were denoted *A*, *CP* and *B* in the order of their mobilities. In the negative limb, however, two boundaries only were visible, the mobilities corresponding to fractions *A* and *B*. The *A* peaks were sharp in both limbs, and comparison of the mobilities given for *A* in Table 3 with those found by Blix (1940) showed the *A* component to be chondroitin sulphate. This conclusion was afterwards confirmed by isolation of the component using the large apparatus of Tiselius (1938). The peaks due to substance *B* were sharp in the negative limb, but rather diffuse in the positive limb, and owing to the difficulty in measuring the position of the diffuse peak

the observed average values of mobility for *B* were subject to some variation. The observed values, however, were near to those recorded for the purified

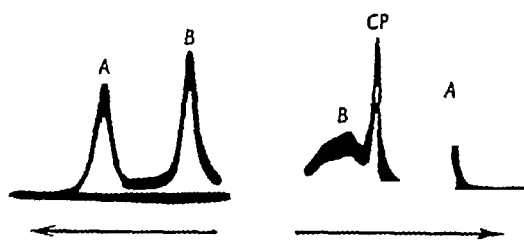


Fig 2a Electrophoresis pattern of chondromucoid in phosphate NaCl buffer at pH 8.5. The preparation was obtained by extracting cartilage powder with phosphate-NaCl solution (pH 6.9) at 60°. Left, negative, descending, right, positive, ascending

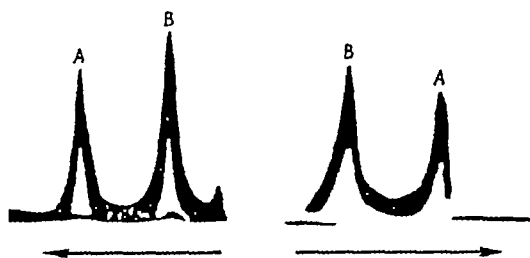


Fig 2b Electrophoresis pattern of chondromucoid after heating at 100° for 30 min. in phosphate NaCl solution of pH 6.88. Left, negative, descending, right, positive, ascending

protein component of the mucoid (given in Table 4), and for this reason the peaks *B* were identified with the protein. The component *CP* gave a very sharp peak in the positive limb, but within the pH range indicated in Table 3 the boundary was absent in the

negative limb. Although the sharpness of the peak facilitated mobility readings, its position varied rather considerably in different preparations and the mobility values recorded in Table 3 show some scatter. At pH 5.25 the peak *CP* showed in both limbs. At this acid reaction the preparations were usually rather opalescent and it was observed that after the current had been passed for some time opalescence was largely confined between the two *CP* boundaries.

The reduction of viscosity on heating the cartilage extracts has already been reported, and Fig 2(b) shows the electrophoresis pattern obtained with a phosphate NaCl extract (pH 6.88) of cartilage that had been heated at 100° for 30 min. in the same buffer in which the extract was made. In heated preparations the *CP* peak was invariably absent, and the two components *A* and *B* appeared with symmetrical peaks in both limbs. Table 3 shows the values for mobility of the two components in phosphate-NaCl buffers of pH 6.88 and 5.33. The time and temperature of heat treatment required to eliminate the component *CP* was found in all cases to be sufficient to complete the first rapid stage in the reaction leading to reduction of viscosity of the extracts, whether the heat treatment was carried out in phosphate-NaCl buffers of varying pH or in aqueous acetic acid solution. It thus appears probable that the component *CP* contributes significantly to the viscosity of the unheated solution. This conclusion is borne out by the extreme sharpness of the *CP* peak and also by the opalescent appearance associated with the complex, both of which observations indicate a particle of considerably greater size than either of the other two components in the system.

Table 3 Electrophoretic pattern of cartilage extract

Exp no	Buffer	pH (0°)	Mobilities (average from both limbs, $u \times 10^5$)		
			Chondroitin sulphate	CP component	Protein
			Unheated extract		
25	Phosphate (μ , 0.02) + NaCl (μ , 0.18)	5.25	-10.54	-5.64	-1.06
21	Phosphate (μ , 0.02) + NaCl (μ , 0.18)	5.89	-10.90	-5.8*	-1.94
22	Phosphate (μ , 0.02) + NaCl (μ , 0.18)	6.93	-11.35	-2.34*	-1.36
19	Phosphate (μ , 0.02) + NaCl (μ , 0.18)	7.86	-11.68	-4.32*	-1.31
26	Glycine (μ , 0.02) + NaCl (μ , 0.18)	9.01	-11.76	-4.04*	-1.48

Extract heated at 100° for 30 min. in phosphate NaCl (pH 6.88) before dialysis against appropriate buffer

35	Phosphate (μ , 0.02) + NaCl (μ , 0.18)	6.88	-12.35	—	-1.12
36	Phosphate (μ , 0.02) + NaCl (μ , 0.18)	5.33	-12.4	—	-1.22

* The boundary appeared in the positive limb of the cell, but was absent from the negative limb

Electrophoresis of the protein component The protein component isolated by extraction of the dry mucoid with 90 % (w/w) phenol solution was electrophoretically homogeneous over a pH range 3.6–7.0. The mobility readings in a series of buffers of total ionic strength 0.20 are recorded in Table 4. The boundaries in both limbs of the cell were sharp and symmetrical and the pH mobility curve obtained by plotting the data in Table 4 was

DISCUSSION

The work described in the experimental section shows that the intercellular matter of hyaline cartilage of beef septum nasi is composed almost exclusively of chondroitin sulphate and proteins of the collagen group, the relationship between these substances having been examined by degradation of the tissue material in a series of controlled steps.

Table 4 *Mobility of the protein isolated from chondromucoid*

Exp no	Buffer	pH	Mobilities ($u \times 10^5$)		
			$u + ve$	$u - ve$	Average
28	Glycine (μ , 0.02) + NaCl (μ , 0.18)	3.58	+2.29	+2.59	+2.44
30	Acetate (μ , 0.02) + NaCl (μ , 0.18)	4.29	+1.19	+1.19	+1.19
31	Acetate (μ , 0.02) + NaCl (μ , 0.18)	5.11	-0.31	-0.31	-0.31
32	Acetate (μ , 0.02) + NaCl (μ , 0.18)	5.53	-0.58	-0.47	-0.53
29	Phosphate (μ , 0.02) + NaCl (μ , 0.18)	6.95	-0.85	-0.75	-0.80

generally of the shape found for gelatin (Moyer & Moyer, 1940). The isoelectric point of the protein in acetate NaCl buffer (μ , acetate, 0.02, μ , NaCl, 0.18) was 4.86. This is close to the value (4.9) found by Moyer & Moyer for the isoelectric point of electrolyzed commercial gelatin adsorbed on quartz or collodion particles, and since the solubility properties, gel formation and analytical composition of the protein were similar to those of authentic gelatin, the protein must be regarded as having originated from collagen.

Electrophoresis of gelatin prepared from the collagenous residue remaining after extraction of the cartilage with alkaline $CaCl_2$ solution The gel-forming protein prepared by heating the collagenous residue in an autoclave was similar in analytical composition to authentic gelatin, and showed a single sharp boundary in the electrophoresis apparatus. The mobility readings given by the protein fitted the mobility pH curve obtained with the samples of gelatin isolated from the chondromucoid (Table 4). It thus appears that, apart from a small insoluble residue (comprising less than 6% of the intact tissue), the ground substance of cartilage is a structure built up from no more than two clearly recognizable chemical individuals, chondroitin sulphate and collagen, the process of successive degradation described in this work may therefore be looked upon as leading to the destruction of a collagen-chondroitin sulphate complex, and ultimately to the complete degradation of the collagen structure to gelatin and the liberation of chondroitin sulphate in a degraded form.

The extreme sensitivity of collagen to increase in temperature led to the view that the collagen structure of cartilage would suffer modification at lower temperatures than does the chondroitin sulphate, and experiment showed that if the temperature of an aqueous suspension of powdered cartilage was raised to a point at which thermal contraction of the collagen would be expected to take place (60–70°) the treatment resulted in the release of a highly viscous, water-soluble mucoid containing chondroitin sulphate in a yield representing some 60% of the amount originally present in the tissue. The use of salt solutions, particularly those of the divalent alkaline earths, had the expected effect in reducing both the temperature of contraction of the collagen of rat tail tendons and the temperature required to release chondromucoid from cartilage to approximately the same degree.

The analytical composition of the mucoid extracted from cartilage remained remarkably constant over a series of extraction experiments in which a number of solvents and salt solutions were used, provided the lowest effective temperature was employed, but the yield of chondroitin sulphate obtained was usually no more than 60–70% of that present in the tissue. If higher temperatures were used, the mucoid had a lower viscosity and contained an excess of protein. Extraction with 10% calcium chloride solution at pH 11 gave yields of 90–95% of the ester-bound sulphur originally present in the tissue, but here again, the viscosity of the extracted mucoid was low.

The water soluble mucoid was examined in the electrophoresis apparatus of Tiselius over a considerable pH range and, in addition to the boundaries due to the protein and the acid polysaccharide, a third boundary, due to a complex between the two, was observed over the whole range covered by the experiments. From pH 9.0 to 5.8 the third boundary (designated *CP*) appeared in the positive limb only, but at pH 5.3 it showed its presence in both limbs and at this acid reaction the solution had a distinct opalescence which appeared to be confined between the two *CP* boundaries. The substance responsible for the *CP* boundary was heat labile and a further stage in the process of degradation was carried out by heating the mucoid at 80–100° for 10–30 min when it no longer showed the presence of the third boundary in the electrophoresis apparatus and the protein and polysaccharide components migrated independently. The disappearance of the *CP* boundary on heating the mucoid was coincident with a considerable fall in the viscosity of the solution, and since the sharpness of the *CP* boundary indicated a particle of considerable length, it seems probable that the complex contributed significantly to the viscosity of the unheated solution. The failure of the component to appear as a distinct boundary in the negative limb at hydrogen-ion concentrations near neutrality may be due to the instability of the complex except in the presence of an excess of chondroitin sulphate. Such mass action effects are common in antigen antibody reactions and reveal their presence in zoning phenomena.

A similar position has been revealed by the work of Chargaff, Ziff & Moore (1941), who examined the electrophoretic behaviour of heparin when mixed with serum albumin or dialyzed plasma. A third component was observed (designated by these authors the *C component*), which had a mobility intermediate between that of serum albumin and the acid polysaccharide. In this case, however, the *C component* was more clearly discernible on the descending side than on the ascending side, which usually showed only a broadening of the albumin peak. Some preparations of heparin failed to produce the *C component*, but in this case the heparin peak appearing on the descending side was many times larger than on the ascending side. Chargaff and his colleagues also examined the effect of chondroitin sulphate on human plasma proteins, but no evidence of complex formation was obtained. However, a highly purified specimen of chondroitin sulphate was used and it may have been somewhat degraded in the process of purification.

Since the complex formation of heparin with serum albumin is of considerable importance in the anticoagulant activity of the former it was considered of interest to assay the activity of 'undegraded' chondroitin sulphate as an anticoagulant.

However, it was found that both the mucoid itself and a preparation of chondroitin sulphate obtained from the mucoid by separation in the large apparatus of Tiselius (1938) had less than 2% of the activity of a commercial preparation of heparin.

Complex formations between proteins and chondroitin sulphate at reactions more acid than the isoelectric point of the protein have been studied in some detail by Meyer *et al* (1937), who show that the mucins produced are in fact true salts, and are formed in stoichiometric proportions. The present demonstration of reversible complex formation at alkaline reactions, however, indicates association of a different type wherein the protein and the polysaccharide are both negatively charged. The character of the bonds involved in a union of the latter type is unknown, but provided the particle size of the components is sufficiently great, polar association is not excluded since, except under very strongly alkaline conditions, the more strongly basic centres of the protein molecule are still charged positively.

It is known that native collagen suffers a loss of ammonia on conversion into gelatin, and there is evidence that in its untreated condition collagen has an isoelectric point higher than that of gelatin prepared from it (cf Ames, 1944). It is, therefore, probable that conditions in the intact tissue are different from those obtaining in the extracted mucoid. However, since complex formation occurs between chondroitin sulphate and protein in the mucoid at hydrogen-ion concentrations within the physiological range, it seems reasonable to presume that it also occurs in the intact tissue. This assumption provides an answer to a point that has frequently been debated. The early workers, in particular Mörner (1889), held that chondroitin sulphate occurred in the tissue as an alkali metal salt, while Meyer *et al* (1937) were of the view that the tissue contains a salt between chondroitin sulphate and protein. It now appears that the position lies between the two extremes, and that the strongly acid sulphate groups are most probably held in combination with some of the basic residues of the protein, the net charge of the complex being adjusted mainly by a competition between alkali metal ions and hydrogen ions for carboxylic acid residues in both protein and polysaccharide.

It has not proved possible to secure the liberation of mucoid from the tissue by any treatment sufficiently mild to avoid modifying collagen, and it is therefore clear that the mucoid, particularly as regards its protein moiety, should be looked upon as an artifact. However, the circumstance that chondroitin sulphate retains its capacity of complex formation in the water soluble system resulting from degradation of the tissue affords some insight into the structure of the tissue itself. If our knowledge

of the chemical behaviour of the polysaccharide is combined with the results of histological investigations the structure may be visualized as a network of collagen fibrils, in some places organized into parallel bundles to form microscopic fibres and in others relatively disorganized and heavily cross-linked by association with chondroitin sulphate. The extraction experiments also suggest that the protein of the mucoid springs from the 'disordered' collagen of the cementing substance rather than from the ordered fibres.

This view of the structure of the tissue is supported by the recent observations of Cohen (1942), who showed that many proteins of plant origin having a molecular weight greater than 10^5 were precipitated by heparin, chondroitin sulphate and hyaluronic acid, and in some cases gave rise to extremely long particles often of paracrystalline form. Large concentrations of the colloidal anion were required to effect the precipitation, but only small quantities of the polysaccharides were carried down by the crystalline precipitates.

These facts invite speculation as to the part played by chondroitin sulphate in the orientation of collagen in developing connective tissue. In tissue cultures and healing wounds the fibres arise from fibroblasts which migrate into the medium or the exudate from the wound, the fibres afterwards becoming oriented into fibre bundles, finally adopting the characteristic form of true collagenous tissue. It may be that the typical structure of collagen in cartilage and connective tissue generally is the result of a process similar in character to the production of the paracrystals observed by Cohen (1942) in which chondroitin sulphate, acting as a multivalent anion, cements together the protein molecules to form fibrous macromolecules and eventually fibre bundles. It is noteworthy that even the most regular white connective tissue, that in tendon, always contains appreciable amounts of mucoid.

SUMMARY

1 The intercellular substance of bovine nasal cartilage is composed almost exclusively of two major components—collagen and chondroitin sulphate.

2 A soluble mucoid may be extracted from dried cartilage powder after a short heat treatment with water at 60–70°.

3 The temperature required to liberate mucoid is reduced in the presence of inorganic salts, alkalis and formamide, and the conditions necessary to secure extraction in good yield are generally those which give rise to thermal contraction of collagen.

4 The mucoid contains chondroitin sulphate and a protein derived from the degradation of collagen. Prepared under the lowest effective temperature conditions, the mucoid behaves in the electrophoresis apparatus of Tiselius as an equilibrium mixture of chondroitin sulphate, protein and a complex formed between the two.

5 The association of chondroitin sulphate with protein in the mucoid occurs over the range pH 5–9 within which both the protein and the acid polysaccharide are negatively charged. The complex formation is of a different type from the salt formation known to occur below pH 4.85.

6 On further heating, the mucoid loses its capacity to form complexes at pH 5–9 and suffers a rapid reduction in viscosity.

7 The part played by such complex formation in the intact tissue is discussed and it is suggested that chondroitin sulphate has an important role in the organization of collagen in developing connective tissue.

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The Influence of Bactericidal Agents and of the Absence of L-Ascorbic Acid on the Accumulation of Hydroxyphenyl Compounds in the Large Intestine of Guinea Pigs after the Consumption of High Doses of L-Tyrosine

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In a previous communication (Painter & Zilva, 1947) it was shown that very much larger quantities of hydroxyphenyl compounds* were found in the large intestine, mainly in the caecum, 3 or 5 hr after the ingestion of high doses of L tyrosine when guinea pigs were depleted of L ascorbic acid, than when they received cabbage *ad lib* with the basal diet. It was further observed that a similar accumulation in the large intestine could be found when sulphaguanidine was administered to guinea pigs subsisting on the basal diet with cabbage *ad lib*. In this paper experimental evidence is given which affords an explanation of this phenomenon, and observations have been made which are of fundamental theoretical interest and have possibilities of therapeutic application.

EXPERIMENTAL

Technique For details of experimental animals and diet, see Penney & Zilva (1946). For methods of collection of urine, preparation of tissues for analysis, determination of total hydroxyphenyl compounds ('tyrosine'), and L-tyrosine, see Painter & Zilva (1947).

The samples of sulphaguanidine, succinylsulphathiazole and phthalylsulphathiazole used were commercial medicinal products.

Administration of the bactericidal agents In the most intensive experiments of long duration (8-15 days), the compounds were administered as aqueous suspensions in quantities varying from 0.1 to 0.3 g, the doses being adjusted from day to day so as to prevent a loss in weight of the animals. In the case of the experiments of shorter duration (3 days) daily graded doses of 0.05, 0.1, 0.2 and 0.3 g of sulphaguanidine were administered to the respective groups. Experiments have proved that guinea pigs on a diet containing cabbage *ad lib*, even after dosage with sulphaguanidine for 8 days, were fully saturated in respect of L ascorbic acid.

* Hydroxyphenyl compounds are usually determined by methods which are based on Millon's reaction. A negative reaction is then taken to indicate rupture of the benzene ring, although in reality it only indicates the disappearance of the phenolic group. For the sake of greater accuracy we abstain in this communication from referring to the rupture of the benzene ring in cases in which the Millon's reaction is absent.

RESULTS

The 'tyrosine' content of the large intestine of guinea pigs after the administration of L tyrosine under different dietetic conditions

Table 1 summarizes the results with sixteen groups, each of 12 guinea pigs. All the animals were killed 3 hr after the administration of 0.5 g of L-tyrosine and the contents of the large intestine, comprising the caecum, colon and rectum, were immediately analyzed for hydroxyphenyl compounds by Folin & Ciocalteu's method (1927). The values thus obtained are calculated as 'tyrosine'. No significant quantities of these compounds were found in the faeces passed during the 3 hr between the administration of the L tyrosine and slaughter. References in the text to animals depleted for a certain time signify the time which elapsed since the removal of the cabbage, previously administered *ad lib*, from the basal scorbutic diet. The statistical treatment of the data was carried out as described by Snedecor (1937) for the determination of the *t* value.

The mean value for 'tyrosine' obtained with the animals receiving cabbage *ad lib* in addition to the basal scorbutic diet during the pre-experimental period (group 1) was very low compared with that obtained with the animals which were depleted for 14 days (group 6). Group 1 also has a wide variation of the individual values. No other group, with the possible exception of group 16, showed this characteristic. This is particularly evident when the high ratio of the standard deviation to the mean is compared with similar ratios in the other groups. This observation is in accordance with expectation, if it be assumed that the quantity of 'tyrosine' found in the large intestine is controlled by the quantity of cabbage present there when the administered L tyrosine reaches the large intestine in considerable quantities. The amount of the cabbage present would in such a case depend on the quantity eaten and on the time when it was consumed and this would vary with the individual animal. In all the other groups the conditions were more under the control of the experimenter and thus the fluctuations were not so marked.

Table 1 *The 'tyrosine' content of the large intestine of guinea pigs 3 hr after the administration of 0.5 g L-tyrosine to animals previously maintained under various dietetic conditions*

Group no	Addition to basal diet	Days	Treatment on day of L-tyrosine dosing	Mean 'tyrosine' content (expressed as mg L-tyrosine)	S.D.	$\frac{S.D.}{\text{Mean}} \times 100$
				24		121
1	Cabbage <i>ad lib</i> in the pre experimental period	0	—	—	29	—
2	Nil	1	—	94	39	42
3	Nil	3	—	134	51	38
4	Nil	5	—	104	49	47
5	Nil	8	—	106	30	28
6	Nil	14	—	159	46	29
7	25 mg L-ascorbic acid daily	6	—	65	32	49
8	Nil	1	25 mg L-ascorbic acid with the L-tyrosine	86	41	48
9	Nil	1	100 mg L-ascorbic acid with the L-tyrosine	58	31	53
10	Nil	14	100 mg L-ascorbic acid with the L-tyrosine	88	46	52
11	Nil	6	100 mg L-ascorbic acid with the L-tyrosine	85	39	46
12	Nil	14	25 mg L-ascorbic acid with the L-tyrosine	108	53	49
13	Nil	14	100 mg L-ascorbic acid 1 hr before, with, and 1 hr after the L-tyrosine	76	47	62
14	Nil	14	Cabbage <i>ad lib</i> immediately after the L-tyrosine	92	35	38
15	Nil	14	100 mg L-ascorbic acid with the L-tyrosine and cabbage <i>ad lib</i> immediately after	98	24	25
16	Nil	14	Cabbage <i>ad lib</i> 2 hr before the L-tyrosine, 100 mg L-ascorbic acid with the L-tyrosine and cabbage <i>ad lib</i> after	29	26	90

That the disappearance of the 'tyrosine' from the large intestine depends on the constant presence of cabbage is seen from the fact that after the removal of the cabbage from the diet for 24 hr (group 2), the quantity of 'tyrosine' present in the large intestine became significantly higher than in group 1 ($t = 4.98$), but it still remained significantly lower than that found after 14 days' depletion (group 6, $t = 3.73$). Unfortunately, an attempt to find out how soon after the removal of the cabbage from the diet the maximum intestinal content of 'tyrosine' might be reached was not completely successful as experiments carried out 3, 5 and 8 days after the removal of the cabbage from the diet (groups 3-5) yielded values which were not always significantly different among themselves, and sometimes not even

so when compared with the mean obtained after 14 days' depletion (cf groups 3 and 6, $t = 1.26$). This is possibly due to the great individual variation in the time during which the unaltered cabbage remains in the lower reaches of the intestinal tract.

The first attempt to ascertain whether the degradation of the phenolic group of the L-tyrosine in the large intestine was connected with the vitamin C content of the cabbage was made by administering daily 25 mg of synthetic L-ascorbic acid in solution for 6 days to guinea pigs maintained on the basal scorbutic diet, the last dose of the vitamin being given with the L-tyrosine. It was found (group 7) that the mean of the contents of this group was of the same order as that of group 2 (animals depleted for

24 hr), but was, nevertheless, significantly higher than that of group 1 which received cabbage *ad lib* ($t=3.29$). The essential point, however, is that the means of groups 3-6 which were depleted for 3, 5, 8 and 14 days respectively ($t=3.97, 2.31, 3.24$ and 5.81 respectively) were markedly higher. In other words, the presence of L-ascorbic acid *per se* is capable of producing a speedier disappearance of the phenolic group of the 'tyrosine' in the intestine. It is of interest to note that after 1 day's depletion the 'tyrosine' values could not be lowered by the administration of 25 or 100 mg of L-ascorbic acid with the L-tyrosine dose (*vide* groups 8 and 9). Yet after 6 or 14 days' depletion the 'tyrosine' values could be lowered to that obtained after 1 day's depletion by the administration with the L-tyrosine of 25, 100 and 300 mg (in three successive doses of 100 mg) of L-ascorbic acid (*vide* groups 10-13).

Various attempts to attain the low 'tyrosine' values observed in the case of guinea pigs on a diet containing cabbage *ad lib* were made. This was accomplished only when cabbage was offered *ad lib* to guinea pigs 2 hr before the administration of 0.5 g of L-tyrosine with 100 mg of L-ascorbic acid and subsequent cabbage *ad lib* (group 16). On the other hand, the administration to guinea pigs depleted for 14 days of (a) three successive doses of 100 mg of L-ascorbic acid, one before, one with and one after the dose of L-tyrosine (group 13), (b) of cabbage *ad lib* with the L-tyrosine (group 14) and (c) of 100 mg of L-ascorbic acid in addition to cabbage *ad lib* with the dose of L-tyrosine (group 15), only lowered the large intestine 'tyrosine' values to values approximating that of the mean of the group of animals depleted for 1 day (group 2). It will be noted that in group 16 the individual values are as widely distributed as in group 1, probably for the same reason.

The evidence in this series of experiments definitely supports the view that the consumption of vitamin C favours the disappearance of the phenolic group of L-tyrosine or of the hydroxyphenyl degradation products of this compound from the intestine. The higher efficiency of cabbage as an agent in this process of degradation as compared with L-ascorbic acid in solution cannot be fully explained at present. Two possible explanations are worth considering. One is that during the transit of the cabbage in the alkaline section of the intestine the vitamin is more protected, and that in the large intestine there is a gradual release of the L-ascorbic acid. In consequence, the total quantity of the vitamin is more efficiently utilized in the reaction in question than when the L-ascorbic acid is in solution, and is more accessible for destruction through other agencies. Alternatively, the cabbage, apart from its physical condition, may contain another sub-

stance which conduces to the breakdown of L-tyrosine in the intestine. We incline to the former view.

The influence of bactericidal agents on the degradation of L-tyrosine in the intestines of guinea pigs

The results of the above experiments strongly indicated the possibility that the accumulation of 'tyrosine' in the large intestine of the depleted guinea pigs was the consequence either of a change in the intestinal flora which was caused by the absence of L-ascorbic acid or of a change in the capacity of some of these organisms to metabolize the phenolic group of L-tyrosine and its degradation products. The obvious approach to the elucidation of the problem was, therefore, to study the influence of bactericidal agents known to modify the bacterial flora of the intestine. The action of sulphaguandine, succinylsulphathiazole and phthalylsulphathiazole was accordingly investigated with this point in view. In each case it was found that these agents when administered *per os* to animals receiving cabbage *ad lib* produced, as in the case of the depleted animals, an accumulation of 'tyrosine' in the large intestine, mainly in the caecum. A detailed and comparative study of guinea pigs receiving cabbage *ad lib* and sulphaguandine as the bactericidal agent on the one hand, and depleted guinea pigs on the other, has, however, revealed some differences between the changes brought about by the presence of the bactericidal agent and the absence of L-ascorbic acid from the diet. These differences, as will be seen later, did not, however, militate against the hypothesis that the absence of L-ascorbic acid and the presence of the bactericidal agent were capable of modifying the intestinal flora of the guinea pig so as to interfere with their degradation of the L-tyrosine present there.

The results of this group of experiments are summarized in Tables 2 and 3. The outstanding fact which emerges from Table 2 is that 3 hr after the administration of L-tyrosine there is a great accumulation of 'tyrosine' in the large intestine of the depleted guinea pigs and of the animals which received cabbage *ad lib* and sulphaguandine, when compared with the content found in the case of the animals receiving only cabbage *ad lib*. Even a daily dose of 0.1 g of sulphaguandine given for 3 days previous to the administration of L-tyrosine already produced this effect. The data in Table 2 disclose a further point which, apart from its general interest, has a bearing on the present problem. It will be seen that the concentration and contents of the hydroxyphenyl compounds in the tissues of the entire intestinal tract in the groups of the depleted animals and of the animals receiving cabbage *ad lib* were much higher than in the case of the groups which were dosed with sulphaguandine. In the

Table 2 *The distribution of 'tyrosine' in the body and urine of guinea pigs maintained on diets containing cabbage ad lib with and without sulphaguanidine, and that of guinea pigs on a scorbutic diet 3 hr after the administration of 0.5 g L tyrosine*

Daily dose of sulphaguanidine No of days No of animals Found in tissues	Mixed diet containing cabbage												Scorbutic diet None 14 6			
	None			0.1-0.3 g 12-15			0.1 g			0.2 g				0.3 g		
	Content (mg)	Conc (mg/ 100 g)		Content (mg)	Conc (mg/ 100 g)		Content (mg)	Conc (mg/ 100 g)		Content (mg)	Conc (mg/ 100 g)			Content (mg)	Conc (mg/ 100 g)	
Stomach tissue	Mean S D	1.9 0.8		Trace	Trace		0.7 0.33	23		0.7 0.58	25		2.0 0.8	67.3		
Stomach contents	Mean S D	6.8 5.4		3.8 4.1	—		20.0 28.3	—		15.1 14.8	—		7.0 7.7	—		
Small intestine tissue	Mean S D	3.7 3.1		Trace	Trace		1.2 1.18	10		0.9 0.5	8		5.4 1.8	40		
Small intestine contents	Mean S D	3.0 1.7		Trace	—		Trace	—		2.6 2.2	—		2.8 0.8	—		
Large intestine tissue	Mean S D	3.3 3.3		Trace	Trace		1.0 0.84	8		1.1 0.64	9		4.1 2.0	39.2		
Large intestine contents	Mean S D	21.1 33.2		150 38.1	—		103 74.9	—		60.5 18.9	—		149 60.3	—		
Blood	Mean S D	7.7 5.2		Trace	Trace		2.1 1.6	10		2.6 1.6	13		11.6 3.9	58		
Liver	Mean S D	2.7 2.3		Trace	Trace		Trace	Trace		Trace	Trace		5.2 1.2	41.6		
Kidney	Mean S D	1.7 2.0		Trace	Trace		Trace	Trace		Trace	Trace		2.0 0.9	75.7		
Muscle	Mean S D	6.5 1.7		Trace	Trace		Trace	Trace		Trace	Trace		29.7 16.5	57		
Urine	Mean S D	11.1 12.05		7.0 3.54	—		0.6 0.56	—		6.0 3.58	—		36.1 21.6	—		

Table 3 *Summary of the distribution of 'tyrosine' (expressed as mg L tyrosine) in the body and urine of guinea pigs maintained on diets containing cabbage ad lib with and without sulphaguanidine, and that of guinea pigs on a scorbutic diet, after the administration of 0.5 g L tyrosine*

Daily dose of sulphaguanidine	Mixed diet containing cabbage												Scorbutic diet None								
	None			0.1-0.3 g		0.1 g			0.2 g			0.3 g		0.05 g							
	0			12-15		8		3			24			3		3					
No of days	3			24		3		24			3			24			3		24		
Time after L tyrosine dose (hr)	5			7		12		6			6			6			6		4		
No of animals	30.9			154		21.3		123			Not done			78.2			143.8			Not done	
Contents of intestinal tract + faeces	27.0			Trace		Not done		5.0			Not done			5.3			3.0			Not done	
Tissues	11.1			7.0		24.7		0.6			18.7			4.8			2.7			22.6	
Urine	69.0			161.0		46.0		128.6			18.7			88.3			149.5			22.6	
Total	89			68		91		74			98			82			70			95	
Percentage not accounted for	89			97		97		97			97			97			95			95	

latter animals, although the size of the dose and the time during which it was administered did not influence the content of 'tyrosine' in the large intestine to an appreciable extent, it did, nevertheless, influence the content and concentration of the hydroxyphenyl compounds in the walls of the intestinal tract. In the animals dosed intensively (0.1–0.3 g sulphaguanidine for 12–15 days) only traces of 'tyrosine' could be found in the intestinal tissues, but low concentrations, bearing a roughly inverse proportion to the dose, could be observed in the animals treated less drastically with the bactericidal agent (0.1, 0.2 and 0.3 g sulphaguanidine for 3 days). The latter 'tyrosine' values were, however, considerably lower than those found in the case of the animals receiving no sulphaguanidine. The difference in the 'tyrosine' content and concentration between the animals dosed with the sulphaguanidine and the other guinea pigs is even more marked in the case of the blood, liver, kidney and muscle. All these differences could be explained by an impaired passage of the 'tyrosine' into the blood stream brought about by the consumption of the bactericidal agent.

This view was strengthened by further evidence. In Table 3 are given the 'tyrosine' contents of (a) the intestinal tract and faeces, (b) the tissues, and (c) the urine of all the animals 3 hr (summarized from Table 2) and 24 hr after the administration of the dose of L-tyrosine. It is seen that while the greater part of the 'tyrosine' in the tissues and large intestine found 3 hr after the administration of the L-tyrosine to the depleted guinea pigs was eliminated by the kidney during the following 21 hr (169 out of 219 mg), only about one eighth was accounted for in the urine of the sulphaguanidine treated guinea pigs. This fact taken in conjunction with the observation that the tissues of these animals, as mentioned above, contained little or no 'tyrosine' when compared with the tissues of the guinea pigs which did not receive sulphaguanidine, supports the view that the consumption of sulphaguanidine greatly impaired the passage of the 'tyrosine' into the blood stream. That some of these compounds did pass into the circulation is seen from the fact that the mean of the 'tyrosine' content of the urine values of the animals receiving sulphaguanidine for 12–15 days was 24.7 ± 15.6 mg (Table 3). This value was significantly higher than the mean we have previously obtained from a control group of 12 guinea pigs not receiving L-tyrosine (6.3 ± 4.1 mg, $t = 3.95$). Furthermore, we have found that, by the prolonged administration of L-tyrosine to guinea pigs receiving sulphaguanidine, 'tyrosine' values reaching 60 mg/day could be attained. Therefore, although some of the 'tyrosine' did enter the system, the best part of it seems to have disappeared from the gut, but at a very much slower rate than in the case of guinea

pigs receiving cabbage *ad lib* without sulphaguanidine. In the case of these animals only 57.9 mg of 'tyrosine' out of the 500 mg of L-tyrosine received was present in the large intestine and in the tissues 3 hr after the administration of L-tyrosine against 154 mg present, entirely in the large intestine, in the case of guinea pigs which received 100–300 mg of sulphaguanidine daily for 12–15 days. After a further 21 hr not a trace was found in the intestines and tissues in the former group of animals, whilst in the latter 21.3 mg 'tyrosine' was still present in the intestinal contents and in the faeces. The evidence indicates that the bactericidal agent had significantly diminished the intestinal bacterial population which is capable of attacking the phenolic group in L-tyrosine, and in consequence the phenolic group of the L-tyrosine or of the hydroxyphenyl degradation products was destroyed to a smaller extent during the first few hours than in the case of the animals on the same diet which did not receive sulphaguanidine.

DISCUSSION

The evidence of this investigation leaves little doubt that the accumulation of 'tyrosine' in the large intestine 3 hr after the administration of L-tyrosine in guinea pigs on a diet not containing cabbage is due to the absence of L-ascorbic acid *per se* and that the accumulation of 'tyrosine' in the gut of the depleted animals is primarily responsible for the incomplete breakdown of the phenolic group in some of the animals receiving a massive dose of L-tyrosine. The reason why the administration of cabbage is more effective than L-ascorbic acid in obviating the accumulation of 'tyrosine' in the large intestine is not quite clear, but in all probability it is due to the greater protection of the L-ascorbic acid when the cabbage is passing through the alkaline region of the small intestine and to the gradual release of the vitamin from the plant tissue. It was further seen that a similar accumulation of 'tyrosine', but without the appearance in the urine of an excess of hydroxyphenyl compounds, can be produced by the administration to guinea pigs of bactericidal agents such as sulphaguanidine, succinylsulphathiazole and phthalylsulphathiazole even when the animals consume cabbage *ad lib*. In order to obtain a clear understanding of the problem it is necessary to correlate the present results with those described by us in a previous communication (Painter & Zilva, 1947).

In the first investigation we demonstrated that when a high enough dose of L-tyrosine was injected in depleted guinea pigs intraperitoneally as glycyl-L-tyrosine, i.e. when the concentration of L-tyrosine in the blood was raised to a certain critical level, there was an increase in the amount of the total hydroxyphenyl compounds eliminated by the kidney and *p*-hydroxyphenylpyruvic acid was excreted in the

urine at the same time * The phenolic group in a part of the L tyrosine was, therefore, not broken down in the system. Very much higher doses of glycyl L-tyrosine had to be injected into animals receiving cabbage to bring about this incomplete degradation of the L tyrosine, evidently because of the much higher concentration of L ascorbic acid in the blood of these animals. It was, therefore, not unwarranted to assume that a critical concentration of 'tyrosine' in the blood, similar to that obtained by injection of high doses of glycyl L-tyrosine, could also be attained by permeation from the large intestine of depleted guinea pigs. The accumulation in the lower reaches of the intestinal tract caused by the absence of L ascorbic acid from the diet of these animals must, therefore, be considered primarily responsible for the partial degradation of the phenolic group in the large dose of L tyrosine. The fate of the 'tyrosine' accumulated in the large intestine of the guinea pigs which received cabbage and sulphaguanidine was different. It will be recalled that the intestinal tissues, blood, liver, kidney and muscle did not contain any appreciable quantities of 'tyrosine', and that only about one eighth of the accumulated 'tyrosine' was accounted for in the urine. This strongly suggested that little of these hydroxyphenyl compounds entered the blood stream and that the highest concentration thus reached was below the critical concentration necessary to produce an abnormal degradation of the compounds in question attained in the depleted guinea pigs. Consequently, no *p* hydroxyphenylpyruvic acid was formed under these conditions, not even when L-tyrosine was administered on several successive days. Since, however, the greater part of the accumulated 'tyrosine' disappeared within the next 21 hr (vide Table 3) it may be assumed that these compounds have been slowly destroyed in the intestine, as compared with the animals which received cabbage without sulphaguanidine, especially as some 'tyrosine' was, under these circumstances, still present in the intestinal contents and in the faeces. We are, therefore, faced with the following outstanding facts: (a) A quick destruction of 'tyrosine' in the case of the guinea pigs receiving L ascorbic acid, since the amount present in the large intestine after 3 hr was small, and although this entered freely into the blood stream containing 'saturated values' of L-ascorbic acid it could not reach the critical concen-

tration above which the system was incapable of metabolizing these compounds normally. Only 3% of the 0.5 g of L tyrosine was accounted for after 24 hr, the remainder having been destroyed. (b) A much slower destruction of the 'tyrosine' with a resultant accumulation in the large intestine after 3 hr in the case of the guinea pigs receiving cabbage and sulphaguanidine. The bulk of this was slowly destroyed in the lower reaches of the intestine during the following 21 hr. In this case 4-9% of the consumed L tyrosine was accounted for (Table 3), most of it being destroyed in the intestine. (c) A slow destruction of the 'tyrosine' in the depleted animals with an accumulation in the large intestine. In this case, however, the hydroxyphenyl compounds enter the circulation freely, and the concentration in the blood is such that in the presence of little ascorbic acid the phenolic group cannot be degraded in all the 'tyrosine', and at the same time some phenolic compounds are produced which are evidently intermediate in the degradation of the L-tyrosine. After 24 hr 41% of the L-tyrosine was accounted for in the urine as hydroxyphenyl compounds (Table 3).

If the possibility of a very rapid passage into the blood stream is excluded, the evidence appears to justify the assumption that the L-tyrosine in the intestine of the guinea pig is normally quickly destroyed by intestinal organisms and that the rate of this destruction can be slowed down to a considerable extent either by administering bactericidal agents or by withholding L ascorbic acid from the diet. In the former case the explanation is fairly clear, the bactericidal agent diminishes the population of the intestinal organisms including those destroying L tyrosine. In the latter case other possibilities may have to be considered. The presence of L ascorbic acid may favour organisms, capable of attacking the phenolic group, which are suppressed in the absence of the vitamin. The vitamin may even act as a necessary link in a chain of reactions concerned in this degradation. The literature on the subject does not throw much light on the problem. The action of various organisms on L-ascorbic acid has been investigated by several workers (Stepp & Schröder, 1935, Kendall & Chunn, 1938, Esselen & Fuller, 1939, Young & James, 1942, Young & Rettger, 1943). Certain strains of these organisms under the requisite conditions can oxidize L ascorbic acid, mostly beyond the reversible stage, but some, such as *Escherichia coli*, which is actually capable of reducing dehydro L ascorbic acid, can act in a protective capacity. Fortunately, according to Crecélius & Rettger (1943), the normal flora of the guinea pig intestine is relatively simple, consisting mainly (about 80%) of a non sporulating rod form of *Lactobacillus*. The remainder of the flora is composed of yeasts and of soil and air bacteria. The investigation of the action of these organisms on phenolic

* In our description of the method of determination of *p* hydroxyphenylpyruvic acid (Painter & Zilva, 1947) we stated inadvertently that the solution of the compound in 2.5% metaphosphoric acid used for the calibration curves was allowed to remain 30 min. after being adjusted to pH 9.0. This should have read that the solution of *p* hydroxyphenylpyruvic acid was adjusted to pH 9.0, allowed to remain for 30 min., after which metaphosphoric acid was added so that the final concentration was 2.5%.

amino acids in the presence and in the absence of L-ascorbic acid may supply information not only of fundamental theoretical interest but also lead to therapeutic applications. The observation that some bactericidal agents are capable of modifying under these abnormal conditions the passage of 'tyrosine' into the blood stream is also of fundamental theoretical and practical interest. As was seen, this impairment of the passage was only partial, since some of the hydroxyphenyl compounds have been shown to enter the blood stream. It may also be noted that the guinea pigs receiving the bactericidal agent were found to be saturated in respect of L-ascorbic acid. This problem requires further investigation.

SUMMARY

1 Three hours after the administration of 0.5 g of L-tyrosine more hydroxyphenyl compounds were found in the large intestine, particularly in the caecum, of guinea pigs which were depleted of L-ascorbic acid and in that of guinea pigs receiving sulphaguanidine, succinylsulphathiazole or phthalylsulphathiazole with cabbage *ad lib* than in guinea pigs receiving vitamin C in the form of cabbage or L-ascorbic acid in solution without the bactericidal agents.

2 A number of experiments are described in which the influence of the mode of administration and of the quantity of the added L-ascorbic acid was investigated, and it is concluded that L-ascorbic acid as present in cabbage is more effective than as the synthetic compound in solution.

3 A great part of the hydroxyphenyl compounds present in the large intestine of the depleted guinea pigs 3 hr after the administration of the L-tyrosine

was found in the urine of the animals 21 hr later. Only a small portion of these accumulated compounds was present in the urine of the guinea pigs receiving cabbage *ad lib* and sulphaguanidine.

4 The concentrations of hydroxyphenyl compounds in the walls of the intestine, the blood, the liver, the kidney and the muscle were much higher in the depleted animals and in the animals receiving cabbage *ad lib* without sulphaguanidine than in the corresponding tissues of animals receiving the bactericidal agent.

5 Excluding a very rapid passage into the blood stream, it may be assumed that the lower content of hydroxyphenyl compounds in the large intestine of the animals receiving cabbage *ad lib* or synthetic L-ascorbic acid in solution without sulphaguanidine is due to the influence of the intestinal bacterial flora which is modified by either the absence of L-ascorbic acid from or the presence of bactericidal agents in the diet of the guinea pigs. In the first case, owing to the free passage into the circulation, the concentration in the blood reaches levels which are too high for these hydroxyphenyl compounds to be metabolized normally. In the second case the impaired passage into the blood stream brought about by the consumption of the bactericidal agents prevented the production of such a critical concentration of hydroxyphenyl compounds in the circulation, and the greater part of these compounds was destroyed slowly during passage along the large intestine.

6 The fundamental and practical potentialities of these observations are stressed.

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Pipette for Use with the Van Slyke-Neill Manometric Apparatus

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In the use of the Van Slyke Neill manometric apparatus, the accurate measurement of reagents maintained free from dissolved air is much facilitated by use of the special pipette illustrated (Fig. 1). This pipette allows 50 ml reagent to be kept out of contact with air for an indefinite period and for any required quantity to be accurately measured into the apparatus without any possibility of coming into contact with air.

with the zero mark on *B* and the top of *E* should be approximately level with the lowest graduation on *B*. Tubes *D*, *F* and *C* should be of capillary bore throughout. In those models of Van Slyke's apparatus with a standard ground socket at the top of the reaction chamber, *F* should be fitted with a corresponding standard cone, otherwise the tip of *F* is fitted with a piece of rubber as usual in the use of Ostwald pipettes. The pipette should be built as compactly as possible, otherwise the weight of mercury in *A* and *E* will be liable to fracture the tubing. If it is so built the apparatus is reasonably sturdy, but a stand with three clips to support the pipette when not in use is an advantage, especially if several pipettes are required.

Operation

Filling pipette (a) *Filling with mercury* To fill the pipette open *X* to connect *A* to *E*, and *Y* to connect *C* to *F*. Pour mercury in through *A* until *E* is full and mercury flows from *F*. Close *X* and *Y* and add about 15 ml more mercury to *A*. Open *X* to connect *A* to *B* until mercury reaches the zero mark in *B*, then close *X*. Pour mercury into *D*, open *Y* to connect *D* to *F* until mercury flows from *F*.

(b) *Introduction of reagent* Free the reagent from air in the Van Slyke apparatus in the usual manner. Place a small quantity of mercury in the cup above chamber *G*, insert the tip of *F*. Raise the levelling bulb of the Van Slyke apparatus to the upper ring, open *Z* to connect *G* to *F*, open *Y* to connect *F* to *C* and *X* to connect *E* to *A*. The solution will flow into *E* displacing mercury into *A*. When all the reagent is in the pipette or the mercury is almost entirely driven from *E*, close *X*, *Y* and *Z*, remove the pipette, open *Y* to connect *D* to *F* so that mercury fills the bore of *F*.

Addition of measured amount of reagent to the Van Slyke apparatus Place the tip of *F* in the cup above chamber *G* of the Van Slyke apparatus (the cup should contain a few drops of mercury). Open *X* to connect *B* to *E* and *Y* to connect *C* to *F*. Turn *Z* slowly until the mercury in *B* indicates that the required volume of reagent has entered chamber *G*. Close *X*, open *Y* to connect *D* to *F* until mercury flows into *G*, close *Y* and *Z*.

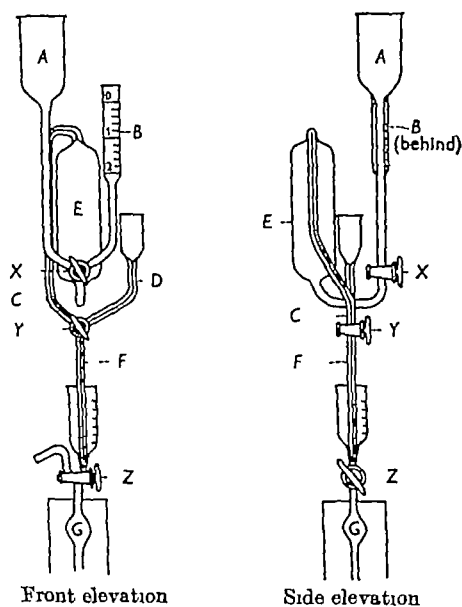


Fig 1 Pipette for use with the Van Slyke Neill manometric apparatus

Construction

The pipette is built round two 3 way stopcocks *X* and *Y*, with branches at 120° and with an angle of 120° in the bore so that any two branches may be connected. Stopcock *Y* should have capillary bore connexions. Bulb *E* and cup *A* should have about 50 ml capacity. *B* is a calibrated tube, for most purposes a capacity of $2 \text{ ml} \times \frac{1}{10}$ is convenient since it is unusual to measure greater volumes of reagent into the chamber of the Van Slyke apparatus, but, if required, the capacity of this tube may be greater. The bottom of cup *A* should be approximately level

To set the pipette for use again, open *X* to connect *A* and *B* and allow mercury to rise to the zero mark in *B*.

It is not usually necessary to empty the pipette. If a different reagent is to be put into it, the aqueous contents may be removed by placing the pipette on the Van Slyke apparatus, opening *X* to connect *A* to *E* and *Y* to connect *C* to *F* and drawing them down into *G*. The fluid is then expelled from *G* to waste, and *G* refilled with water which is passed up into the pipette, this process being repeated until the pipette is clean. Should it become necessary to

empty mercury out of the pipette this is most conveniently done by holding the pipette face downwards over a suitable wide container and withdrawing the plug of cock *X*.

SUMMARY

A special pipette is described for use with the Van Slyke manometric apparatus. It allows storage of reagents out of contact with air for indefinite periods and measurement of any required quantity of reagent into the manometric apparatus without its coming into contact with air.

Synthesis of Glutamic Acid in Animal Tissues*

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In earlier experiments (Krebs & Henseleit, 1932) it was noted that slices of rat liver, suspended in a saline medium containing ammonium salts, often removed more ammonia from the medium than could be accounted for by the formation of urea. The present work arose from a study of the reactions other than the synthesis of urea, which cause a disappearance of ammonia in liver tissue.

EXPERIMENTAL

Analytical methods. Urea was determined manometrically with urease (Krebs & Henseleit, 1932; Krebs, 1942), NH_3 was steam distilled *in vacuo* according to Parnas and Heller, as described by Krebs (1935), and measured photoelectrically after nesslerization. Amino N was determined with Van Slyke's (1929) manometric nitrous acid method, after deproteinization with trichloroacetic acid and removal of NH_3 . Glutamic acid was determined according to Gale (1945) with the decarboxylase of *Clostridium welchii*, strain SR 12. Succinic and α -ketoglutaric acids were determined by the succinic dehydrogenase method (Krebs & Eggleston, 1948a), acetoacetate with aniline citrate (Edson, 1935), and oxaloacetate with $\text{Al}_2(\text{SO}_4)_3$ (Krebs & Eggleston, 1945).

Tissue preparations. Homogenates were made in the glass apparatus of Potter & Elvehjem (1936), and in a similar instrument made from stainless steel. The medium used for the dilution of the tissue was 'phosphate saline without Ca' (Krebs & Eggleston, 1940) or phosphate bicarbonate saline without Ca. Lower rates of reaction were found when Ca was present in physiological concentrations. A medium simulating the ionic composition of liver cells (containing 6 ml. of 0.1M CaCl_2 , 18 ml. of 0.1M MgCl_2 , 15 ml. of 0.1M K_2HPO_4 , 10 ml. of 0.1M KH_2PO_4 , 10 ml. of 0.155M KCl

and 41 ml. of 0.155M NaCl) gave rates of about 25% and, when Ca was omitted, of about 90% of those in 'phosphate saline without Ca'. The homogenates were used as freshly as possible. On storage their activity, as measured by their capacity to form glutamic acid, fell fairly rapidly, e.g. by c. 50% when kept in the refrigerator for 24 hr.

Slices were suspended in phosphate saline or phosphate bicarbonate saline.

Adenosine triphosphate (ATP). Ba ATP (64.7 mg.) was dissolved in 3.3 ml. of 0.1N HCl. Ba was precipitated by the addition of 0.1 ml. of M Na_2SO_4 and the solution was neutralized with 0.15 ml. of 2N NaOH. Water was added to 5 ml. The precipitate was removed by filtration or centrifugation. The solution contained 0.02M ATP.

General. The material was incubated in conical Warburg vessels. The substrates were placed in the side arm and added to the tissue preparation at zero experimental time, usually when the manometers were attached to the water bath. The temperature was 40°. All substrate concentrations mentioned in the tables refer to the final concentrations in the medium. In anaerobic experiments yellow P was placed in the centre well. Quantities of metabolites are expressed in μl . and rates of metabolic processes in the usual *Q* values. When homogenates were used the dry weight for calculating the *Q* values was assumed to be 30% of the fresh weight in the case of liver and 20% in the case of brain cortex, kidney cortex or muscle.

To stop the metabolic processes in homogenates after a specified time the flasks were detached from their manometers and 1 ml. of 2N HCl/4 ml. suspension was added as quickly as possible. If the analyses could not be carried out immediately the solutions were stored in the refrigerator. Of the acidified suspension usually 1 ml. was used for the determination of glutamic acid, 1 ml. for NH_3 , 3 ml. for succinic acid. If a larger amount of material was required for further analyses, duplicate Warburg cups were set up and the contents were combined after completion of the incubation.

* Preliminary account, *Nature, Lond.*, 159, 808 (1947).

Homogenates often contained significant amounts of NH_3 , glutamic acid and other metabolites. These initial concentrations were determined in a sample acidified with 2N HCl (0.25 vol.) at the start of the experiment.

RESULTS

Excess of ammonia consumption over urea formation. Examples showing the quantities of ammonia removed by rat liver slices in relation to urea formation are shown in Table 1. The amount which cannot be accounted for by the urea formation varied with the presence of other substrates and was of the same order of magnitude as those converted into urea. In these experiments no ornithine or citrulline was added. In the presence of these substances the excess of ammonia consumption over urea formation was usually small.

rat liver was only a small fraction, usually less than 10%, of the rate observed in slices, unless ornithine or citrulline was added. For example, Q_{urea} was 7.1 in slices and 0.25 in homogenized rat liver (substrate 0.01M ammonium chloride). Similar results have been reported by Cohen & Hayano (1946). Homogenates, however, utilize ammonia if suitable substrates are present (Table 2). Of the substrates tested α ketoglutarate had the greatest effect on the utilization of ammonia, citrate was slightly less effective, pyruvate and oxaloacetate still less. No appreciable effects were found with fumarate, lactate, glucose and acetoacetate. With α ketoglutarate the rate observed in this experiment was of the same order as in slices.

Effect of tissue dilution. In the preceding experiments 1 part of the tissue was diluted with medium to about 7 parts of suspension. Dilution to 15 parts

Table 1 Urea formation and ammonia consumption in rat-liver slices

(Slices suspended in 4 ml. phosphate saline. Incubation 1 hr.)

Substrates	Tissue (mg)	Urea (μl)	Q_{urea}	NH_3 left (μl)	NH_3 removed (μl)	Q_{NH_3}	Amount of NH_3 (μl)	
							Accounted for by urea	Not accounted for by urea
None	12.60	13	1.03	Nil	Nil	0	—	—
NH_4Cl (0.005M)	13.30	53	3.98	250	-180	-13.52	80	100
+ L-lactate (0.02M)	8.65	35.5	4.10	358	-72	-8.32	45	27
+ pyruvate (0.02M)	8.50	34	4.00	328	-102	-12.00	42	60
+ oxaloacetate (0.02M)	13.80	57	4.13	250	-180	-13.04	88	92
+ glucose (0.02M)	11.80	57	4.84	285	-145	-12.29	88	57

Table 2 Removal of ammonia in rat liver homogenate

(One part of liver homogenized with 4 parts of phosphate bicarbonate saline, 3 ml. diluted with 1 ml. of substrate solution or saline, 5% CO_2 , 95% O_2 , 40°, incubation 20 min., concentration of other substrates 0.01M, NH_4Cl added, 430 μl , NH_4Cl found in suspension at start, 63 μl .)

Substrate added (in addition to NH_4Cl)	NH_3 found (μl)	Change in NH_3 (μl)	Q_{NH_3}
α Ketoglutarate	116	-377	-6.3
Citrate	266	-227	-3.8
Pyruvate	318	-175	-2.9
L-Lactate	482	-11	-0.2
Acetoacetate	469	-24	-0.4
Fumarate	486	-7	-0.1
Glucose	512	+19	+0.3
Oxaloacetate	395	-98	-1.6
None	512	+19	+0.3

Ammonia utilization in liver homogenates. It was thought advantageous for the present purpose to work under conditions in which the urea synthesis was suppressed whilst other reactions involving the utilization of ammonia still proceeded. This object was largely realized by the use of homogenized liver. After homogenization the rate of urea synthesis in

decreased the initial rate of ammonia removal in proportion to the tissue concentration, in other words Q_{NH_3} remained constant. In the subsequent experiments a final tissue concentration of 1 part of tissue in 9 parts of medium was used unless otherwise stated.

Effect of oxygen and of saline media. Rat liver homogenates, in the presence of α ketoglutarate, also remove ammonia anaerobically. The data in Table 3 show that the anaerobic rate can even be higher, by about 15%, than the aerobic rate. The presence of bicarbonate and carbon dioxide, which is of importance for the formation of urea, did not affect the rate of the reaction responsible for the removal of ammonia in the present experiment.

Since anaerobic conditions reduce the number of reactions that occur in tissue preparations, and thus simplify the interpretation of results, the majority of the following experiments were carried out anaerobically.

Formation of glutamic acid. Determinations of amino N in the solutions of the experiment recorded in Table 2 showed an increase in amino N approximately equivalent to the loss of ammonia. The fact that α ketoglutarate was the most effective substrate in removing ammonia and forming amino N

Table 3 *Aerobic and anaerobic removal of NH₃ in the presence of α ketoglutarate in rat liver homogenate*

(One part of liver homogenized with 4.5 parts of saline, each cup contained 3 ml of homogenate and 1 ml of additional solution (substrate or saline). Incubation 30 min. The homogenate contained 90 μ l. NH₃ per cup from internal sources at the start of the experiment. The data refer to 4 ml of suspension.)

Medium	Phosphate saline				Phosphate bicarbonate saline			
	O ₂		N ₂		5% CO ₂ in O ₂		5% CO ₂ in N ₂	
Gas								
NH ₄ Cl added (μ l)	0	914	0	914	0	914	0	914
α -Ketoglutarate added (μ l)	0	1792	0	1792	0	1792	0	1792
NH ₃ found after incubation (μ l.)	71	264	124	242	102	322	150	276
Change in NH ₃ (μ l.)	-19	-650	+34	-678	+12	-592	+60	-638
Same corrected for suspension without added NH ₄ Cl		-631		-712		-604		-698
Q_{NH_3}		-7.7		-8.7		-7.4		-8.5

suggested that the reaction under investigation was the reductive amination of α ketoglutarate, and the solutions were therefore tested for glutamic acid by Gale's (1945) method. It will be seen from Table 4 that the quantities of ammonia removed and

Table 4 *Formation of glutamic acid in rat-liver homogenate*

(One part of liver, 9.5 parts of phosphate saline, N₂, 40°, 40 min incubation. The corrected values were obtained by deducting the values found in the absence of substrate.)

Substrates added (final concentration)	None	α Ketoglutarate (0.01 M)
		NH ₄ Cl (0.01 M)
NH ₃ change (μ l) (observed)	+45	-318
(corrected)	—	-363
Glutamic acid (μ l.) (formed)	+40	+395
(corrected)	—	+355

glutamic acid formed agree within the limits of error if corrections are made for changes in the tissue which occur without the addition of substrates. Other examples showing this agreement are found in Tables 5 and 6, in some experiments the formation of glutamic acid was rather smaller than the removal of ammonia.

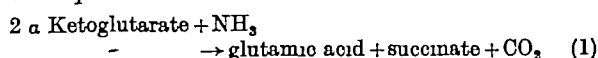
Formation of succinic acid The reductive amination of α -ketoglutarate is necessarily coupled with an equivalent oxidative process. Previous observations in this laboratory (Krebs & Cohen, 1939) suggested that the oxidative decarboxylation of α -ketoglutarate might be the equivalent reaction. The experimental data recorded in Table 5 show

Table 5 *Anaerobic formation of succinic acid*

(Conditions as in Table 4.)

Substrates added	None	α Keto- glutarate (0.01 M)
		NH ₄ Cl (0.01 M)
NH ₃ change (μ l.) (observed)	+58	-322
(corrected)	—	-380
Glutamic acid found (μ l) (observed)	+84	+415
(corrected)	—	+331
Succinic acid found (μ l) (observed)	+18	+394
(corrected)	—	+376

that this is essentially true: the yields of glutamic and succinic acids and the amounts of ammonia used in this experiment are roughly in accordance with the equation

Table 6 *Comparison of changes in NH₃, glutamic acid and succinic acid in liver homogenates*

(Final tissue concentration 10%, α ketoglutarate and NH₄Cl 0.01 M, 'blank' refers to tissue incubated without substrate.)

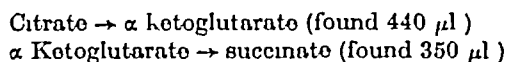
Species	Period of incubation (min.)		NH ₃ (μ l.)	Glutamic acid (μ l.)	Succinic acid (μ l.)
Guinea pig	40	Blank	+42	+95	+35
		With substrates	-287	+375	+222
		Data corrected for blank	-329	+280	+187
Guinea pig	40	Blank	+37	+93	+20
		With substrates	-336	+430	+398
		Data corrected for blank	-373	+337	+378
Rat	60	Blank	+106	+65	+15
		With substrates	-236	+305	+205
		Data corrected for blank	-342	+240	+190
Rat	20	Blank	+21	+17	0
		With substrates	-353	+253	136
		Data corrected for blank	-374	+236	136

In other experiments, however, the yield of succinate was low and the disappearance of ammonia high (Table 6). The former observation was taken to indicate that other oxidative reactions can be coupled with the synthesis of glutamic acid, the latter suggests that additional reactions causing the removal of ammonia occur in liver homogenates.

Effects of various metabolites on the synthesis of glutamic acid. In order to find the other reactions which might be coupled with the synthesis of glutamic acid, various substrates were added together with α ketoglutarate and ammonium chloride under anaerobic conditions. No definite effects on the yield and rate of formation of glutamic acid were found with glucose, glycogen, glucose 1 phosphate, acetate, crotonate, acetoacetate, glycerol, α glycerophosphate, ethanol, stearate and choline. Three sets of substances (Table 7) (a) citrate and the two related tricarboxylic acids, (b) β hydroxybutyrate, (c) fumarate and L-malate increased the rate of glutamic acid synthesis as well as the final yield, which is 50% of the added α ketoglutarate if reaction (1) is the only mechanism leading to glutamic acid. Major inhibitions of both glutamic acid synthesis and ammonia consumption were found with L lactate and hexosediphosphate (Table 8). Acetate and butyrate caused slight inhibitions in some experiments. L Alanine increased, and pyruvate decreased, the yield of glutamic acid owing to transamination.

Examples showing the effects of citrate, β hydroxybutyrate and fumarate in guinea-pig liver are given in Table 7. In this experiment, as in all similar ones,

citrate had the greatest effect. It increased the removal of ammonia and the formation of glutamic acid almost equally without appreciably affecting the formation of succinate. The sum of the α ketoglutarate, succinate and glutamate found exceeded by 440 μ l the amount of α ketoglutarate added. This can be explained by the assumption that citrate was converted into α -ketoglutarate under the conditions of the experiment. The yields and the calculations concerning the oxido-reduction balance are in agreement with the view that the reductive amination of α ketoglutarate (found 782 μ l) was coupled with the following two oxidative reactions



The effect of β hydroxybutyrate was much smaller than that of citrate but was also definite. The yield of glutamate, which in the absence of added substrate does not exceed 50% of the α ketoglutarate (as expected from equation 1), reached 59%, while the yield of succinate showed a slight decrease. The presence of acetoacetate in the solution indicates that the additional oxidative reaction coupled with the glutamate formation was the oxidation of β -hydroxybutyrate to acetoacetate.

In the case of fumarate the yield of glutamate also exceeded 50%, it was 64%. The fact that oxaloacetate appeared indicates that the reaction fumarate \rightarrow oxaloacetate was in this case one of the oxidative equivalents of the glutamate formation. That the amount of oxaloacetate found was re-

Table 7 *Effect of citrate, β hydroxybutyrate and fumarate on the synthesis of glutamic acid in guinea-pig liver homogenate*

(General conditions as in Table 6, incubation 40 min., substrate concentrations all 0.01M except DL β hydroxybutyrate which was 0.02M. Controls showed that in the absence of α ketoglutarate and NH_4Cl citrate yielded no α ketoglutarate, β hydroxybutyrate no acetoacetate and fumarate no oxaloacetate.)

Substrates added	None	α Keto glutarate, NH_4Cl	α Keto glutarate, NH_4Cl , citrate	α Keto glutarate, NH_4Cl , DL β hydroxy butyrate	α Keto glutarate, NH_4Cl , fumarate
NH_3 (μ l.) change	+37	-336	-754	-496	-612
Corrected		-373	-791	-533	-649
Glutamic acid formed (μ l)	+93	+430	+875	+800	+663
Corrected		+337	+782	+507	+570
Succinate formed (μ l.)	+20	+398	+370	+380	+438
Corrected		+378	+350	+360	+418
Acetoacetate formed (μ l)	0	0	0	+210	0
Oxaloacetate formed (μ l.)	0	0	0	0	+38
α Ketoglutarate found (μ l)	0	104	204	49	0
α Ketoglutarate added (μ l.)	0	896	896	896	896
Sum of α ketoglutarate, succinate and glutamic acid recovered (corrected)		819	1336	916	988
Oxido-reduction balance					
Reductions					
Formation of glutamic acid		337	782	507	570
Oxidations					
Formation of succinate		378	350	360	418
Formation of acetoacetate or oxaloacetate			790	570	456
Formation of α ketoglutarate			440	210	38

latively small is not unexpected because of its rapid secondary reactions. Some oxaloacetate must have reacted with glutamate by transamination, hence the amount of glutamate found was smaller than that of the ammonia removed. Another fraction of oxaloacetate probably reacted to form the tri-carboxylic acids, α -ketoglutarate and malate (Krebs, Eggleston, Kleinzeller & Smyth, 1940), hence the sum of α -ketoglutarate and succinate exceeded the amount of α -ketoglutarate added. In all experiments on guinea pig liver the effect of fumarate was of a similar magnitude, in rat liver the effect was smaller, but also marked.

The effects of citrate, β hydroxybutyrate and fumarate suggest that the reductive amination of α ketoglutarate can be coupled with four different oxidative reactions (a) α ketoglutarate \rightarrow succinate, (b) isocitrate \rightarrow α -ketoglutarate, (c) β -hydroxybutyrate \rightarrow acetoacetate, (d) fumarate (or L malate) \rightarrow oxaloacetate.

Examples of the inhibitory effect of lactate, hexosediphosphate, acetate and butyrate are given in Table 8. The inhibition of glutamic acid formation by 0.01 M-L lactate in guinea-pig liver was 73%. The inhibition of the ammonia uptake was of the same order. No explanation for the mechanism of this inhibition can be offered.

Effect of adenosinetriphosphate If the experimental periods were short (less than 10 min) and the tissue concentrations in the homogenate high (10% or more), addition of ATP had little or no effect on the rate of glutamic acid synthesis. In longer experiments with more dilute suspensions, ATP had definite effects in that it delayed the decline in the rate. These effects were larger if sodium fluoride, which inhibits the hydrolysis of ATP, was also present (see Table 9). The effects may be taken to indicate that ATP is a component of the system responsible for the synthesis of glutamic acid.

Effect of substrate concentration As Table 9 indicates, the rate of glutamic acid synthesis in homogenates falls quickly with time. In further experiments, designed to measure the initial rates, a short incubation period of 10 min was therefore chosen. When the concentration of α -ketoglutarate was varied between 0.005 and 0.04 M, the highest rates were found at 0.02 M. Variations of the concentration of ammonium chloride between 0.05 and 0.02 M, with a concentration of α -ketoglutarate of 0.02 M, had no effect on the rates.

Rate of glutamic acid synthesis in homogenates and slices of various tissues Table 10 compares the rates of the synthesis of glutamic acid in homogenates with those in slices of liver and kidney. In the case

Table 8 *Inhibition of glutamic acid synthesis by lactate and other substances in liver homogenates*

(Concentration of α ketoglutarate and NH_4Cl , 0.01 M, N_2 , data refer to 4 ml.)

Species	Period of incubation (min.)	Additional substrates	Glutamic acid found (μL)	NH_4 removed (μL)
Rat	20	—	237	375
		L-Lactate, 0.02 M	68	86
		Na acetate, 0.01 M	180	165
Guinea pig	15	—	201	—
		L-Lactate, 0.01 M	55	—
		Hexosediphosphate, 0.01 M	33	—
		Na butyrate, 0.01 M	150	—

Table 9 *Effects of adenosine triphosphate and NaF on the rate of glutamic acid synthesis in rat liver homogenates*

(Medium phosphate saline, 40°, N_2 , 0.02 M- α ketoglutarate, 0.01 M NH_4Cl . The initial values for glutamic acid in the homogenates have been deducted.)

No	Tissue concentration (%)	Additional substrates added	Glutamic acid formed (μL)	
			10 min.	20 min.
1	10	None	255	263
		ATP, 0.001 M	277	295
		NaF, 0.02 M	223	273
		ATP, 0.001 M, NaF, 0.02 M	281	368
			15 min.	30 min.
2	5	None	99	126
		ATP, 0.001 M	141	166
		NaF, 0.02 M	78	101
		ATP, 0.001 M, NaF, 0.02 M	125	206

Table 10 *Comparison of the rates of glutamic acid synthesis in homogenates and in slices*

(Data for homogenates and slices given in the same horizontal line refer to the same tissue except where an asterisk is added, 0.02M α ketoglutarate, 0.01M NH_4Cl Incubation period for homogenates 10 min, N_2)

Tissue	Slices		
	Homogenate incubation $Q_{\text{glutamic acid}}$	Period of (min)	$Q_{\text{glutamic acid}}$
Liver, rat	12.8	20	1.0
Liver, rat	12.3	20	2.0*
Liver, rat	9.3	30	4.8*
Liver, rat	10.5	30	4.1*
Liver, guinea pig	9.8	20	4.5
Liver, sheep	3.3	15	1.0
Liver, pigeon	15.5	30	2.7*
Liver, pigeon	17.6	—	—
Kidney cortex, rat	7.1	30	4.7*
Kidney cortex, rat	2.0	30	2.7*
Kidney cortex, rat	5.2	—	—
Kidney cortex, sheep	3.3	15	3.2
Kidney cortex, sheep	4.2	15	2.6

of homogenates the values given are corrected for the initial amount of glutamic acid, and with slices for the amount of glutamic acid found on incubation without addition of substrates. It will be seen that in rat liver homogenates $Q_{\text{glutamic acid}}$ was between 9.3 and 12.8, against 1.0–4.8 in slices. For the livers of the guinea pig, sheep and pigeon the rates were likewise much greater in homogenates. On the other hand, the differences between sliced and homogenized material were small in kidney cortex of the rat and sheep.

In slices the rate of glutamic acid synthesis increased with time, in contrast to the fall that occurred in homogenates. Examples are given in Table 11. The accelerating effect of citrate already

reported for homogenates was also found in slices. In the liver and kidney of sheep and rats citrate approximately doubled the rate of synthesis, in homogenates the increase was usually about 50%. The highest rates of glutamic acid synthesis in the presence of citrate were, in terms of $Q_{\text{glutamic acid}}$, 27 in pigeon liver and 20 in rat liver.

Table 11 *Time course of glutamic acid synthesis in sliced tissue*(0.02M α ketoglutarate, 0.01M NH_4Cl , N_2)

Tissue	Time of incubation	$Q_{\text{glutamic acid}}$
Liver, sheep	First 15 min	1.0
	Second 15 min	2.1
Kidney, sheep	First 15 min	3.2
	Second 15 min	6.5

In brain, minced heart muscle and pigeon breast muscle, small but measurable quantities of glutamic acid were always found on incubation with α keto glutarate and ammonium chloride. Addition of citrate usually caused a considerable increase (Table 12). Negligible rates ($Q_{\text{glutamic acid}} < 1$) were found in sheep spleen and guinea pig lung.

Synthesis of glutamic acid in relation to the removal of ammonia in sliced liver. The question arises whether the synthesis of glutamic acid is one of the reactions other than urea synthesis causing a disappearance of ammonia in liver slices. To test this, liver slices were shaken aerobically under conditions similar to those stated in Table 1, and the amounts of ammonia removed, and of urea and glutamic acid formed, were measured (Table 13). Only a small proportion of the ammonia removed which was not accounted for by the formation of urea was recovered as glutamic acid. The proportion of the latter was largest when citrate was the substrate, but even in this case amounted to only 23% of the missing nitrogen. There was no formation of glutamine under

Table 12 *Rate of glutamic acid synthesis in various tissues*(0.02M α ketoglutarate, 0.01M NH_4Cl , 0.01M-citrate, N_2)

Tissue	Substrates added	Period of incubation (min.)	$Q_{\text{glutamic acid}}$
Brain cortex, sliced, sheep	α Ketoglutarate, NH_4Cl	30	0.8
	α Ketoglutarate, NH_4Cl , citrate	30	1.5
Brain cortex, sliced, guinea pig	α Ketoglutarate, NH_4Cl	30	0.9
	α Ketoglutarate, NH_4Cl , citrate	30	2.3
Brain cortex, sliced, guinea pig	α Ketoglutarate, NH_4Cl	30	1.3
	α Ketoglutarate, NH_4Cl , citrate	30	4.8
Brain cortex, sliced, pigeon	α Ketoglutarate, NH_4Cl	30	1.0
	α Ketoglutarate, NH_4Cl , citrate	30	1.4
Brain, minced, sheep	α Ketoglutarate, NH_4Cl	20	0.4
	α Ketoglutarate, NH_4Cl , citrate	20	0.4
Heart, minced, sheep	α Ketoglutarate, NH_4Cl	20	1.4
	α Ketoglutarate, NH_4Cl , citrate	20	2.6
Striated muscle, minced, pigeon	α Ketoglutarate, NH_4Cl	20	1.5
	α Ketoglutarate, NH_4Cl , citrate	20	1.8

Table 13 *Formation of glutamic acid in rat liver slices in the presence of NH_4Cl and other substrates*

(Phosphate saline, O_2 . The data refer to 30 mg (dry) tissue suspended in 4 ml of saline, and are corrected for amounts formed by tissue without added substrate, concentration of NH_4Cl , 0.005M, of other substrates, 0.01M)

Exp no	Substrates added	Period of incubation (min)	NH_3 removed (μL)	NH_3 removed not accounted for by urea (μL)	Glutamic acid found (μL)
1	NH_4Cl	60	167	161	27
2	NH_4Cl	90	96	71	0
	NH_4Cl , citrate	90	51	43	10
	NH_4Cl , glucose	90	9	9	0
	NH_4Cl , fumarate	90	0	0	0

Table 14 *Removal of glutamic acid by rat liver slices*

(Phosphate saline, O_2 . The data refer to 40 mg (dry) tissue suspended in 4 ml. phosphate saline, and are corrected for amounts formed by tissue without added substrate)

Exp no	Substrates added	Period of incubation (min.)	Glutamic acid removed (μL)	Urea + NH_3 formed (as NH_3) (μL)
1	L-Glutamate, 0.01M	60	172	6
	L-Glutamate, 0.01M, NH_4Cl , 0.005M	60	161	51
2	L-Glutamate, 0.005M	90	93	19
	L-Glutamate, 0.005M, NH_4Cl , 0.005M	90	93	40
3	L-Glutamate, 0.005M	90	140	65
4	L-Glutamate, 0.005M	90	74	34

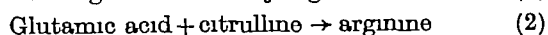
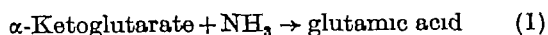
the conditions of the experiments. For the interpretation of this result it is of importance, however, that liver tissue is capable of removing considerable quantities of glutamic acid without forming equivalent quantities of urea or ammonia, as is shown in Table 14. Accumulation in the slices did not account for an appreciable amount of the glutamic acid removed from the medium. The non appearance of glutamic acid is therefore no proof of its non-formation. Glutamic acid may be used up in secondary reactions, and it is therefore possible that the synthesis of glutamic acid accounts for a greater proportion of the missing nitrogen than the data in Table 13 suggest.

DISCUSSION

The occurrence of a synthesis of glutamic acid in animal tissues was first recorded by Euler, Adler, Gunther & Das (1938) and Dewan (1938). The main new contribution of the present work is the measurement of the rate of this reaction under varying conditions, and the observation that the reductive amination of α -ketoglutarate can be coupled with the reaction L-malate \rightarrow oxaloacetate, as an alternative to the three reactions α -ketoglutarate \rightarrow succinate + CO_2 (Krebs & Cohen, 1939), isocitrate \rightarrow α -ketoglutarate + CO_2 (Adler, Euler, Gunther & Plass, 1939), and β -hydroxybutyrate \rightarrow acetoacetate (Dewan, 1938).

Physiological significance of the synthesis of glutamic acid. There are many indications that glutamic acid has a special function in the metabolism

of various cells, though so far little information is available on this point. The rapid synthesis of glutamic acid in liver may serve partly to supply the requirements of this acid for the whole body, but the major part probably reacts by 'transamination', according to Cohen & Hayano (1946), and is thus a link in urea synthesis. The rates of transamination and of glutamic acid synthesis are of the same order and sufficient to account for the formation of urea, the highest value for Q_{urea} observed in rat liver being about 20 (Krebs & Henseleit, 1932) and for $Q_{\text{glutamic acid}}$ about 20. The stage citrulline + $\text{NH}_3 \rightarrow$ arginine of the ornithine cycle can therefore be resolved into the two stages



It has always been obvious that the synthesis of urea through the ornithine cycle, being an endergonic process, must be coupled with exergonic reactions, but no information on the nature of the exergonic reactions was previously available. It is now clear that the oxidative reactions which are coupled with the amination of α -ketoglutarate are components of the system of exergonic reactions supplying the energy for the synthesis of urea.

Several facts indicate that reaction (2) is a complex process. It requires the presence of oxygen (Borsook & Dubnoff, 1941) and is inhibited by low concentrations (0.005M) of malonate (Cohen & Hayano, 1946). The malonate inhibition is abolished

by fumarate (Fahrlander, Favarger, Nielsen & Leuthardt, 1947) This effect of fumarate is a catalytic one, under suitable conditions one molecule of fumarate (or L malate) can cause the formation of many more than five molecules of arginine (Krebs & Eggleston, 1948b) Ratner (1947) suggests that reaction (2) consists of two stages

Glutamic acid + oxaloacetate \rightarrow aspartic acid + α ketoglutarate

Aspartic acid + citrulline \rightarrow L malate + arginine

Whilst the effect of fumarate on the malonate inhibition would be explained by this scheme it is difficult to account for the following four observations on rat liver homogenates (Krebs & Eggleston, 1948b)

(1) Aspartate and citrulline yield arginine very slowly if the conditions are anaerobic, the rate being less than 5% of that observed aerobically with glutamate and citrulline

(2) Aerobically, glutamate and citrulline react about five times faster to yield arginine than aspartate + citrulline

(3) Aspartate removes the malonate inhibition of reaction (2) less effectively than fumarate, the restoration being 50% with 0.005M aspartate against 100% with 0.005M-fumarate

(4) Malonate (0.005M) completely abolishes the interaction between aspartate and citrulline in oxygen, glutamate or α ketoglutarate restores the capacity of liver homogenates to form arginine

Differences between sliced and homogenized material
It would not be surprising to find that the rate of metabolic processes taking place in slices of animal tissues is greatly reduced, even to zero, by homogenization of the tissue, but it is somewhat unexpected to observe the reverse, i.e. a rapid rate in homogenates against an almost negligible rate in slices The first to report such a phenomenon were Cohen & Hayano (1946) working on transamination in rat liver The present observations on the reductive amination of α ketoglutarate in sliced and homogenized rat material are in general similar to those on transamination In both cases there is no major difference in sliced and homogenized kidney, whilst in rat liver the rates in homogenates are about three to fifteen times greater than in slices To explain the relatively low rate of reaction in liver slices one may assume that either the enzyme systems concerned are largely inactive in the intact cell, owing to the presence of intracellular inhibitors, or that permeability barriers prevent the access of the substrates to the enzymes Whatever the explanation of the phenomenon it is clear that major differences exist in the behaviour of kidney and liver cells

SUMMARY

1 A search has been made for reactions, other than urea synthesis, causing a disappearance of added ammonia in isolated liver preparations Homogenization of liver was found to abolish, under certain conditions, the conversion of ammonia into urea, whilst ammonia was still removed when pyruvate, oxaloacetate, α ketoglutarate, or citrate were added The main reaction responsible for the removal of ammonia in homogenates, on addition of the above substrates, was found to be the synthesis of glutamic acid

2 The synthesis of glutamic acid in the presence of α ketoglutarate and ammonia occurred both anaerobically and aerobically, the anaerobic rates being somewhat higher than the aerobic rates

3 The reductive amination of α ketoglutarate by liver can be coupled with the following four oxidative reactions α ketoglutarate \rightarrow succinate + CO_2 (Krebs & Cohen, 1939), isocitrate \rightarrow α ketoglutarate + CO_2 (Adler *et al.* 1939), β hydroxybutyrate \rightarrow acetate (Dewan, 1938), L malate \rightarrow oxaloacetate (not recorded previously)

4 L Lactate and hexosediphosphate, and to a less extent acetate and butyrate, inhibited the formation of glutamic acid from α ketoglutarate and ammonia

5 Adenosine triphosphate delayed the fall in the rate of glutamic acid synthesis which occurred in homogenates when the experimental period exceeded 10 min

6 The rate of glutamic acid synthesis in liver homogenates was three to fifteen times greater than in liver slices In kidney cortex the differences in the rates observed in homogenized and sliced material were small

7 The highest recorded rates of glutamic acid synthesis, in terms of $Q_{\text{glutamic acid}}$, were 20 for rat liver, 27 for pigeon liver, 8 for kidney cortex (sheep, rat), 5 for brain cortex, 3 for heart muscle, 2 for pigeon breast muscle, <1 for spleen and lung

8 Glutamic acid was removed by liver slices without forming ammonia or urea, an indication of the occurrence of unknown reactions in which glutamic acid is utilized.

9 The synthesis of glutamic acid is responsible for a part, but probably not for the whole, of the ammonia which is removed by liver slices and not accounted for by urea formation

10 The physiological significance of the synthesis of glutamic acid is discussed One of the reactions for which glutamic acid is required in liver tissue is presumably the conversion of citrulline into arginine in the course of the synthesis of urea (Cohen & Hayano, 1946)

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The Determination of Quinine Degradation Product in Blood, and its Absorption in the Chick

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In a previous publication (Marshall, 1945a) we reported that quinine degradation product (QDP) showed no antimalarial action against *Plasmodium gallinaceum* in young chicks, when given orally in amounts equivalent to an active dose of quinine. Subsequently, Kelsey, Oldham, Cantrell & Geiling (1946) reported that, when given intravenously, the compound showed one third to one fifth the activity of quinine against chick malaria, and less than one twentieth that of quinine against *P. lophurae* when given orally to ducks. Pointing out that QDP was less toxic than quinine, thus making its therapeutic index nearly equal to that of quinine, these workers disagreed with our view that the metabolism of quinine results in an inherently less efficient drug. They suggested that the reduction of both antimalarial activity and toxicity might be due to a decreased rate of absorption in the host.

In order to test this theory, we decided to compare the concentrations of quinine and QDP attained in the red cells of chicks following oral doses of the compounds, and this necessitated the designing of a method of determination of small quantities of QDP. The fluorescence of QDP in ultraviolet light was too feeble for the determination of very small amounts, though it has been used to determine large concentrations (Knox, 1946). We found, however, that the bromothymol blue method (Marshall & Rogers, 1945) was almost as sensitive with QDP as with quinine.

The degradation product, which is prepared by incubating quinine with rabbit liver suspension, was shown by Mead & Koepfli (1944) to be quinine carbostyryl, i.e. quinine with a hydroxyl group substituted in position 2 of the quinoline ring. This confers phenolic properties on the compound, making it unextractable from sodium hydroxide solution, thus providing a ready method for the separation of QDP from quinine. The degradation product can be extracted from ammoniacal solution (Kelsey, Geiling, Oldham & Dearborn, 1944). We have used the basic principles of this method, but emulsification problems made quantitative extraction difficult. However, by modifying the technique suitably, and carefully controlling the shaking operations, a method giving satisfactory recoveries of small amounts of QDP was produced.

METHODS

Quinine degradation product. This was prepared by incubating quinine with rabbit liver suspension, and extracting and purifying the compound according to the method of Kelsey *et al.* (1944). The melting point of the final product was 250°.

Chicks and dosing. The chicks used were 7–14 days old, and their body weights ranged between 50 and 80 g. A single dose of 200 mg/kg of quinine or QDP base was given into the crop by a catheter tube. The bases were dissolved in a minimum amount of HCl. Pairs of birds from the quinine and QDP groups were killed at suitable intervals by placing

them in an atmosphere of CO_2 . The blood from each pair was withdrawn from the heart and mixed in a graduated tube, heparin and citrate being added to prevent clotting. The tubes were centrifuged at about $6800 \times g$ for 15 min, and the volume of plasma and red cells recorded. The concentration of quinine or QDP was determined in samples of both plasma and red cells.

Determination of quinine Quinine was extracted and determined by the bromothymol blue method, previously described by Marshall & Rogers (1945).

Determination of QDP Add 1 ml. of plasma or red cells to 5 ml. of distilled water in a 100 ml glass stoppered, narrow necked reagent bottle. Add 1 ml. of 5N NaOH and digest in an oven at 100° for 30 min. Cool and add 1 ml. (or sufficient to make the digest acid) of 5N H_2SO_4 , followed by 1 ml. of ammonia (sp gr 0.88). Cool and add 25 ml. of chloroform containing 4% ethanol. Stopper and shake the bottle for at least 1 min, but not too violently, or a persistent emulsion will be formed. It is sufficient to keep the two phases well mixed for the period of 1 min. Stand until the two layers are clearly separated, then centrifuge the bottle gently to promote complete separation. Suck off the upper aqueous layer as cleanly as possible at the water pump. Transfer the chloroform layer to a 6 x 1 in glass stoppered test tube and rinse out the bottle with 5 ml. of distilled water, adding this to the tube. Mix the two layers by inverting the tube several times, centrifuge, and remove the upper layer as before. Repeat the washing of the chloroform layer with a further 5 ml. of distilled water. Shake the chloroform extract with 2.5 ml. of 0.05N HCl for at least 0.5 min, and test the upper layer to ensure that it is still acid. Centrifuge, and transfer the acid layer by means of a test pipette to a 10 ml. flask. Repeat the acid extraction with a further 2.5 ml. of acid, and add it to the first extract. Warm the flask on a sand bath to drive off dissolved chloroform, and continue the bromothymol blue coupling procedure as for quinine.

It should be noted that if QDP is being determined in the presence of quinine, the quinine must first be removed by shaking the NaOH digest repeatedly with ether until a little of the ether extract, when shaken with dilute H_2SO_4 , shows no fluorescence in the acid layer when viewed in filtered ultraviolet light.

RESULTS

The recoveries of known amounts of QDP added to chick blood are recorded in Table 1. Considering the additional technical difficulties, these values com-

Table 1 Recoveries of known amounts of quinine degradation product added to chick blood

QDP added (μg)	QDP recovered (μg)	Percentage recovery
5	6.0	120
5	5.1	102
10	12	120
10	9	90
25	27	108
25	23	92
35	35	100
35	35	100
50	48	96
50	47	94

pare favourably with the recoveries of quinine by the bromothymol blue method (Marshall & Rogers, 1945).

The plasma and red cell concentrations of quinine and QDP up to 7 hr after dosing are shown graphically in Fig 1. The red cell concentration of both compounds was higher than the plasma concentration. The quinine curve was characterized by a sharp peak 1 hr after dosing, followed by a rapid

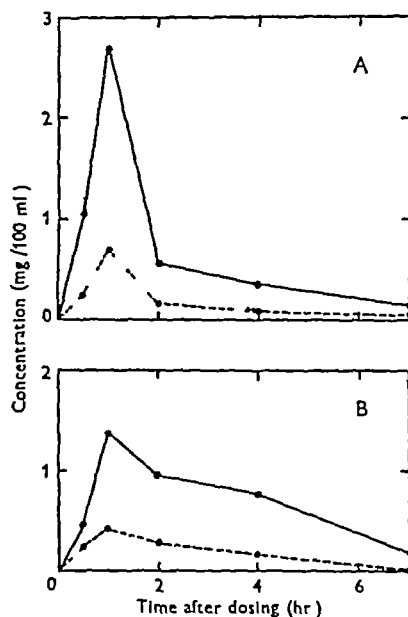


Fig 1 Concentrations of quinine (A) and QDP (B) found in the red blood cells (continuous line) and plasma (broken line) of chicks following oral doses of 200 mg/kg. Each point represents two birds.

decline, which confirms our previous observations on the absorption of quinine (Marshall, 1945b). The red-cell concentration curve for QDP did not show a high initial peak, but the concentration declined less rapidly than with quinine. If the areas enclosed by the respective curves are compared, the QDP concentration over the first 7 hr exceeds that of quinine in the ratio 1.11:1.

DISCUSSION

The results show that, over a period of 7 hr after oral dosing, the total amounts of quinine and QDP appearing in the red cell are approximately the same. In view of the fact that the concentration curve of the degradation product falls off less rapidly than that of quinine, it may be concluded that, on considerations of red cell concentration, QDP has the greater opportunity for being actively anti-malarial than quinine. The observed lower anti-malarial activity of QDP cannot, therefore, be

attributed to poor absorption in the host, but must be due to a specifically inferior activity against the malaria parasite itself

SUMMARY

1 A method for the quantitative determination of quinine degradation product (QDP), sensitive to 5 μ g, is described

2 The red cell concentration of QDP in chicks during 7 hr following oral doses is approximately the same as that of an equal dose of quinine, indicating that the previously observed higher anti-malarial activity of quinine is due to higher specific activity on the parasite, and not to superior absorption in the host

The authors are indebted to Miss C Alexander and Mr R Neville for valuable technical assistance

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The Relationship between Losses in Labile Liver Cytoplasm and Urinary Nitrogen Excretion

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When transferred from an adequate stock diet to a protein-free diet, female rats lose 26 % and male rats 36 % of their liver cytoplasm in 5 days (Kosterlitz, 1944, 1947, Campbell & Kosterlitz, 1946) It has also been observed in many species that under similar conditions the excretion of urinary N gradually falls until a relatively constant value is reached on the fifth or sixth day (Voit, 1866, 1881, cf Kosterlitz & Campbell, 1945-6) The excretion of this extra N appears to be closely correlated with the loss of 'labile' liver cytoplasm

METHODS

Animals Rats of the hooded strain of the Rowett Research Institute were used The females were 16-18 weeks and the males 13-14 weeks old The general management was the same as that described previously (Kosterlitz, 1947)

Diets The protein free diets used were the basal diets of Procedures 1 and 3 described previously (Campbell & Kosterlitz, 1948a) Apart from vitamin supplements, 2 % agar, 3 % salts and 10 % lard, they contained either 60 % potato starch and 25 % sucrose (Exps 1, 3, 4) or 85 % sucrose (Exp 2) Whole liver concentrate was given in Exps 1, 3 and 4 but not in Exp 2, in which the supplements of B vitamins were wholly synthetic (Procedure 3)

Experimental procedure Urine and faeces were collected by the method of Ackroyd & Hopkins (1916) The flasks for the collection of urine were charged with 5 ml of 0.2N H_2SO_4

The funnels were washed down twice daily first with 20 and then with 25 ml. of 0.2N H_2SO_4 , and at the end of each daily period with water The combined urine and washings were made up to 250 ml Urinary N was determined by the micro Kjeldahl method (see Kosterlitz, 1947) In the experiments with sucrose, the whole faeces and, in those with potato starch, suitable samples of the finely ground faeces were ashed by the procedure of Chubnall, Rees & Williams (1943), the period of ashing, however, was reduced to 2 hr after the clearing of the digestion mixture A sample containing about 0.2 mg N was used for distillation

Calculation of the results Each experimental period lasted for 7 days The mean of the daily urinary N excretions on the sixth and seventh days was taken as the basal value 'Extra N' in urine was the difference between the N actually excreted and the basal N All values were expressed as mg/100 g initial body weight In the regression equations, NNS_t stands for labile non glycogen non lipid liver solids (mg/100 g body weight), N_t for labile protein N (mg/100 g body weight) and t for days of protein free diet

RESULTS

Faecal N and basal urinary N (Table 1) The values for faecal N from the second to the seventh day of the protein free diet did not differ significantly from day to day within each experiment and were therefore pooled In the rats which received the protein free diet with 60 % potato starch (Exps 1, 3, 4), the mean daily faecal N values were high because of the

Table 1 *Excretion of 'extra N' in the urine, and loss of protein + nucleic acid N from the liver, during the first 5 days of a protein free diet*

Experimental diet	No and sex of rats	Results/100 g body wt						Loss of 'extra N' in urine
		Initial body wt (mean and range) (g)	Mean daily food intake (g and cal)	Mean daily faecal N (2nd-7th day) \pm s.e. of the mean (mg)	Mean daily basal urinary N (6th and 7th day) \pm s.e. of the mean (mg)	Total 'extra N' in urine (1st-5th days) (mg)	Total loss of protein + nucleic acid N from liver (1st-5th days) (mg)	
1 Protein free with starch following stock diet	4 ♀	230 (216-244)	7.2 (24.5)	20.8 \pm 0.7	8.4 \pm 0.5	53	28	0.53
2 Protein free with sucrose following stock diet	4 ♀	224 (197-262)	6.15 (26.6)	9.0 \pm 0.6	17.1 \pm 1.0	47	28	0.60
3 Protein free with starch following stock diet	4 ♂	318 (308-338)	7.2 (24.5)	21.3 \pm 0.7	9.6 \pm 0.2	41	39	0.05
4 Protein free with starch following one week 54% casein diet	4 ♂	271 (248-287)	7.1 (24.2)	24.5 \pm 1.0	11.7 \pm 0.6	91	59	0.65

In Exp. 4, the liver N on the 5th day of a protein free diet following the stock diet was used for calculation instead of that on the 5th day of a protein free diet following the 54% casein diet, therefore the calculated N loss may possibly have erred on the large side

low digestibility of potato starch (Campbell & Kosterlitz, 1948a), whilst in the rats receiving the protein free diet with sucrose (Exp. 2) the faecal N was low. On the other hand, the reverse holds for the basal urinary N values: they were low in the rats receiving potato starch and high in those receiving the sucrose diet. Thus, the presence or absence of potato starch caused only little variation in the total basal (urinary + faecal) N excretion on the last 2 days of the experimental periods, the daily means were 29.2, 26.1, 30.9 and 36.2 mg/100 g body weight for Exps. 1-4 respectively. The slightly raised value in Exp. 4 can probably be accounted for by the fact that these rats were transferred from a high protein to the protein-free diet.

'Extra N' in urine and loss of protein + nucleic acid N from the liver (Tables 1 and 2). The daily urinary 'extra N' excretions (actually excreted N - basal N) were totalled for the first 5 days of the protein free diet in order to obtain the total 'extra N' excreted during this period. This value showed only small variations in Exps. 1-3, in which male or female rats were transferred from the stock diet (N = 3.2%) to the protein free diet. On the other hand, it was considerably raised in Exp. 4 in which the protein-free diet followed a 54% casein diet.

The losses of protein + nucleic acid N, which may be expected to occur from the liver during the same period, were calculated from the protein + nucleic acid N contents of livers, obtained in earlier experiments (Kosterlitz, 1947; Campbell & Kosterlitz, 1948b). They are summarized in Table 2. The ratios of 'loss of liver N/extra N in urine' indicate that

rather more than half of the 'extra N' was derived from the protein and nucleic acid lost from the liver. If the surprisingly high value of Exp. 3 is included, the mean of the ratios is 0.68, and without it, 0.59. Non protein N and lipid N, amounting to approximately 10% of the protein + nucleic acid N, will further increase the ratios. Thus, even if the high coefficient of variation is taken into consideration, at least 60% of the 'extra N' excreted in the urine during the first 5 days of a protein free diet was accounted for by the N lost from the liver during this period.

Table 2 *Protein + nucleic acid N in livers of rats fed on different diets*

Diet	No and sex of rats	Protein + nucleic acid N (mean and s.e. of the mean) (mg/100 g initial body weight)
Stock	7 ♀	105.9 \pm 2.0
4 days protein free (starch)	4 ♀	78.0 \pm 1.7
7 days protein free (starch)	4 ♀	76.2 \pm 1.7
5 days protein free (sucrose)	5 ♀	78.0 \pm 1.5
Stock	4 ♂	110.0 \pm 2.1
7 days 54% casein	4 ♂	129.6 \pm 5.8
5 days protein free	4 ♂	70.5 \pm 1.6

Daily variations of 'extra N' in urine and of losses of protein and nucleic acid N from the liver (Figs. 1-3). The curves obtained for the daily excretions of total urinary N in Exps. 1, 3 and 4 were not inconsistent

with the results in human beings of Martin & Robison (1922), who found that the excretions of 'extra N' in the urine diminished exponentially during the first few days of a protein free diet. On the other hand, the curve of Exp 2 is rather irregular.

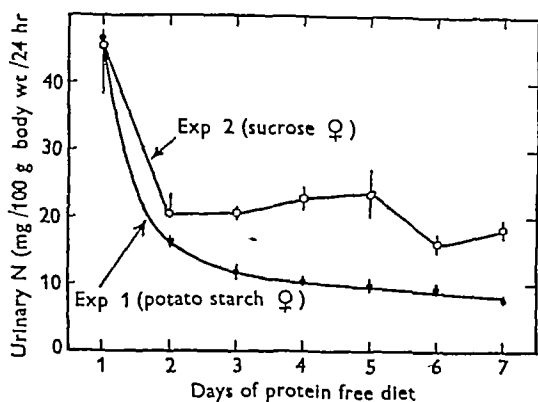


Fig 1 Excretion of total urinary N in female rats fed on a protein free diet (The curves were fitted by eye) The vertical lines represent twice the standard error of the mean and in some instances, in order to avoid confusion, were drawn in one direction only

The daily losses of protein and nucleic acid N from the liver were calculated from the regression equations for labile N (mg/100 g body weight) on days of protein-free diet $N_t = 31.7 (0.33)^t$ for female rats, and $N_t = 42.5 (0.60)^t$ for male rats. These equations were obtained from the regression equations for labile non glycogen, non lipid liver

The values obtained in Exps 1 and 3 for 'extra N'/24 hr in urine and for protein + nucleic acid N lost from the livers in 24 hr are plotted in Fig 3

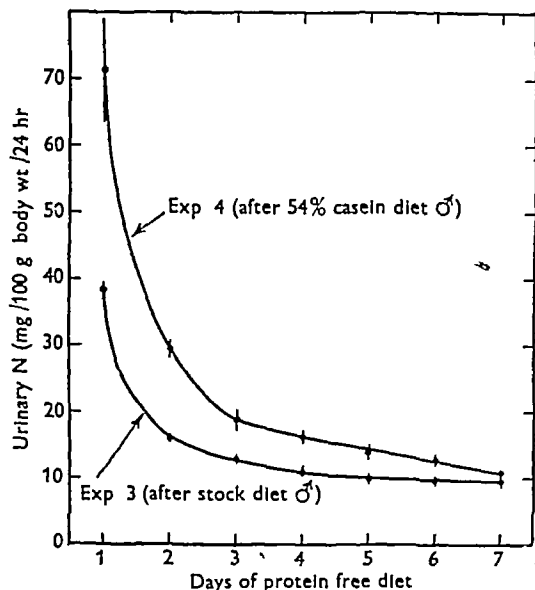


Fig 2 Excretion of total urinary N in male rats fed on a protein free diet (The curves were fitted by eye) The vertical lines represent twice the standard error of the mean

In Exp 1 (female rats) the two curves run close together with the exception of the value for the first day, while they show a considerable discrepancy in

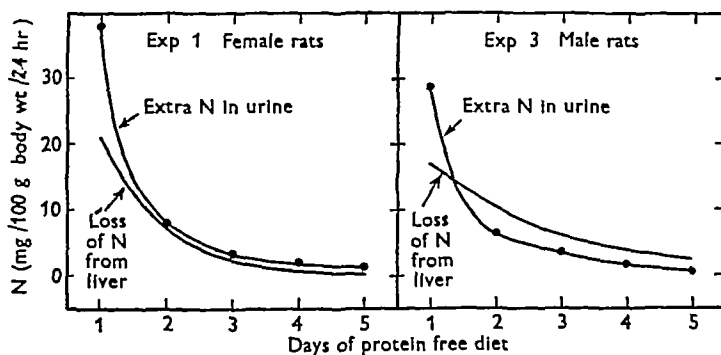


Fig 3 Excretion of 'extra N' and loss of N from the liver (The curves for 'extra N' were fitted by eye and those for loss of liver N were calculated from regression equations)

solids (mg/100 g body weight) on days of protein-free diet $NNS_t = 254 (0.33)^t$ for female rats, and $NNS_t = 352 (0.60)^t$ for male rats. The constants 254 and 352 were multiplied by 0.916×0.136 and 0.885×0.136 respectively, the factors 0.916 and 0.885 convert NNS to protein + phospholipin + nucleic acid, and the factor 0.136 converts the latter to protein + nucleic acid N (Campbell & Kosterlitz, 1948a, c)

Exp 3 (male rats) Whether this difference between male and female rats is significant cannot be decided with the relatively few data at present available

DISCUSSION

The lag in the fall of urinary N to a new equilibrium when dogs were fasted, or when their protein intake was reduced, induced Voit (1866, 1881) to formulate

his theory of 'circulating protein' This was supposed to serve as a reserve pool for protein, which was utilized by the tissues These fundamental observations have been amply confirmed by numerous workers and extended to animals of many species, either fasted or fed on protein free diets Rubner (1911) thought that the 'extra N' excreted during the first few days of a protein free diet was due to 'storage protein', which was retained or lost within 3-5 days after increasing or decreasing the protein intake Storage protein was assumed to be unorganized, and to have as its main function the maintenance of nitrogen equilibrium with an intermittent food intake

The experiments described in this paper, however, indicate that, while a given quantity of 'extra N' appears in the urine, at least 60 %, and probably more, of this amount of N is lost from the liver It is of interest to note that both the losses of nitrogen from the liver (Campbell & Kosterlitz, 1946, 1948a) and the excretions of 'extra N' (Martin & Robison, 1922) diminish exponentially during the first few days of a protein free diet The fact that no other organ loses nitrogen at a rate comparable with that of liver during 2 days of fasting or of a protein free regimen (Addis, Poo & Lew, 1936a, b) supports the view that most of the 'extra N' is derived from the liver

Conversely, Addis *et al* (1936b) found that, in rats which had been transferred from a protein free to a 74 % casein diet, the first definite increase in liver protein occurred as early as 17 hr after the

protein feed Campbell & Kosterlitz (1948a), who depleted rats of their labile liver cytoplasm by a protein free regimen lasting 4 days and then fed them on an 18 % casein diet, found that more than half of their labile liver cytoplasm was restored in 24 hr and that the recovery was virtually complete in 48 hr These findings suggest that the retention of urinary nitrogen during a period of increased protein intake can at least partly be ascribed to the formation of labile liver cytoplasm

SUMMARY

1 An attempt has been made to correlate the excretion of 'extra N' in the urine of rats during the first few days of a protein-free regimen with the nitrogen lost from the livers during the same period

2 At least 60 % of the 'extra N' can be accounted for by the nitrogen derived from the protein, nucleic acid and other nitrogenous substances of the liver

3 These findings make unlikely the older assumption of an unorganized 'circulating' or 'storage' protein which was supposed to be responsible for the appearance of the 'extra N'

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An Improved Diacetyl Reaction for the Estimation of Urea in Blood

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The diacetyl or carbamido reaction of Fearon (1939) has been utilized by Ormsby (1942) and by Barker (1944) for the estimation of urea in biological fluids. Archibald (1945) has used the homologue of diacetyl, α -isomitosopropiophenone (the monoxime of benzoyl acetyl) for a similar method which has the advantage that the colour obtained is red instead of yellow. By analogy with the Barritt (1936) reaction, in which α naphthol is used to enhance the colour obtained from creatine and diacetyl in alkaline solution, it was considered possible similarly to enhance the diacetyl-urea reaction. The observation of Barker (1944) that thymol interfered with this reaction lent support to the hypothesis. Phenols were first tried but produced precipitates. With aromatic amines the colour produced was orange or red and not yellow, whilst diphenylamine and its derivatives produced an intense magenta colour. The reaction with *N*-phenylanthranilic acid (diphenylamine 2 carboxylic acid) has been studied in detail and adapted for the estimation of urea in blood.

EXPERIMENTAL

Effect of added substances on the carbamido reaction

The effect of the addition of various substances to the carbamido reaction was tested in the following manner. To 2 ml of a solution of urea (0.1 mg) 0.25 ml of 3% diacetyl monoxime was added, followed by a few milligrams of the substance under test and finally by 4 ml of 18*N* H_2SO_4 . The solution was placed in a boiling water bath for 10 min, and immediately after removal from the bath 0.25 ml of 1% potassium persulphate was added. The nature of the colour was then observed. With α naphthol and thymol a precipitate was produced which dissolved on the addition of ethanol to give a red coloration. Aniline, α naphthylamine and α naphthyldimethylamine gave a red or orange red colour and clear solutions. With amidopyrin, an immediate violet colour was obtained which faded rapidly and was slowly succeeded by red. Diphenylamine and phenylanthranilic acid gave intense magenta colours.

The nature of this effect was further investigated by examination of the absorption spectra. Those obtained with α naphthyldimethylamine, diphenylamine, and phenylanthranilic acid are given in Fig. 1. That produced by added α naphthyldimethylamine is characteristic of the simple aromatic amines, the spectrum shows two maxima, one in the blue and one in the green, and the intensity of absorption is much less than that for the yellow colour obtained

in the absence of added amine. With the diphenylamine derivatives there is only one absorption band in the green, and the intensity is much greater than that of the original yellow colour, in the case of phenylanthranilic acid more colour was obtained with 0.02 mg of urea than with 0.1 mg using the diacetyl reaction.

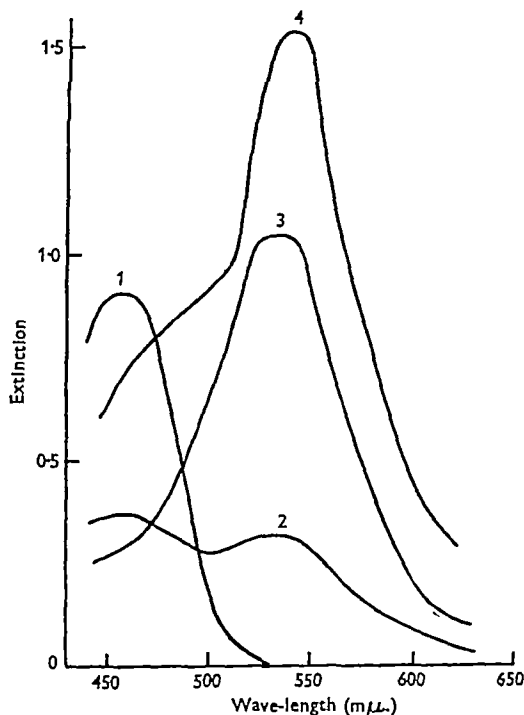


Fig. 1 Absorption spectra of solutions obtained in the diacetyl test for urea: (1) with no added amine, 0.1 mg of urea; (2) with added α naphthyldimethylamine, 0.1 mg of urea; (3) with added diphenylamine, 0.1 mg of urea; (4) with added *N* phenylanthranilic acid, 0.02 mg of urea.

Investigation of reaction conditions with phenylanthranilic acid

Amount of reagents Two molecules of phenylanthranilic acid per molecule of urea were required for maximum colour production, and in order to keep the blank as low as possible minimal amounts were used. The diacetyl monoxime and potassium persulphate could be varied within wide limits without alteration of the colour produced, the amounts eventually chosen were those used by Barker (1944). The concentration of H_2SO_4 for the condensation could be varied between 9 and 14*N* without alteration of the intensity of the colour, although the weaker acid solutions

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were more turbid and the colour faded more rapidly. Here again the conditions of Barker were retained by using 4 ml of 18N H_2SO_4 .

Time of heating The reaction appears to reach completion after 10 min heating in the water bath, no further increase occurs even if the heating is continued up to 30 min (Fig 2)

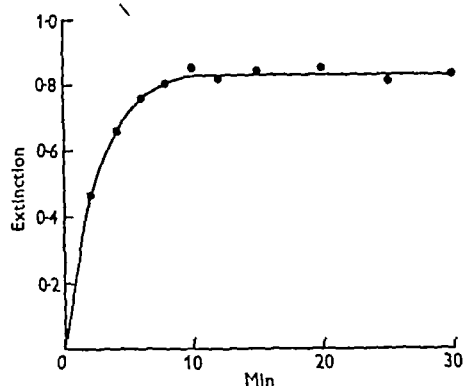


Fig 2 Effect of varying times of heating in water bath on the intensity of colour produced in the improved diacetyl test for urea using *N* phenylanthranilic acid

Development of colour After the addition of persulphate the colour develops rapidly and reaches a fairly steady value in 10–15 min (Fig 3), the colour then remains stable for at least 2 hr. The persulphate must be added as quickly as possible after the tubes are removed from the water bath, otherwise colour production is poor.

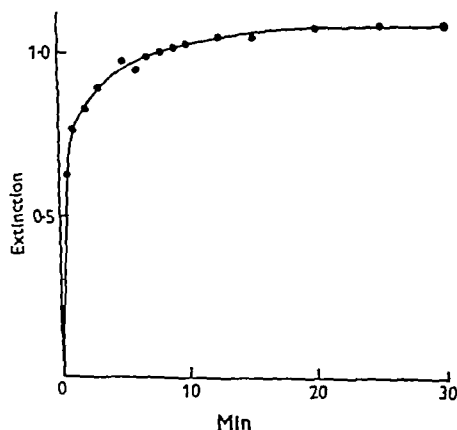


Fig 3 Rate of development of colour after the addition of persulphate, in the reaction between urea and diacetyl in the presence of *N* phenylanthranilic acid.

Calibration curve. Calibration curves have been prepared both with the Lefko photometer and the Spekker absorptiometer (Fig 4). The colour obeyed Beer's law up to a urea concentration of 20 $\mu\text{g}/\text{ml}$, beyond this the readings were erratic possibly due to difficulties in measurement of the high extinctions obtained.

Specificity In specificity this reaction is similar to the carbamido test but with certain important differences,

ammonia, glycine, histidine, tyrosine, cystine, caffeine, uric acid, barbiturates, acetamide, asparagine, creatine, sulphonamides and thiouracil all give negative reactions. Proteins and monosubstituted ureas, e.g. phenylurea, give red colorations. Semicarbazide gives a magenta colour.

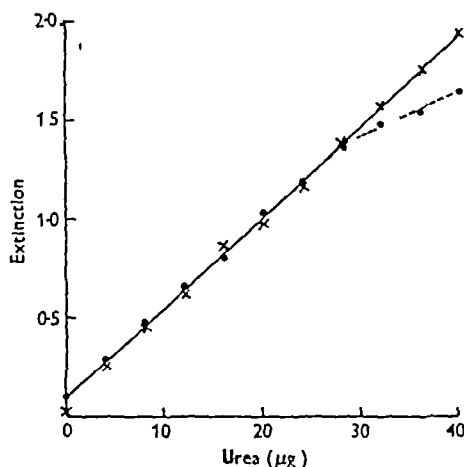


Fig 4 Calibration curve for the improved diacetyl test with known amounts of urea. Dots are values obtained with Spekker photoelectric absorptiometer (spectrum green filter together with calorix heat filter), crosses are results with Leiko visual photometer (filter 530).

similar to that obtained with urea, different from the bright red obtained in the carbamido reaction and of lower intensity. Buret gives a brown colour. Creatinine gives a positive reaction, and this is an important difference between the two tests, but fortunately the sensitivity in this case is only one hundredth of that with urea, so that creatinine will not interfere with the estimation of urea in biological fluids.

BLOOD UREA METHODS

Reagents

Zinc sulphate solution, 2% (w/v)

Sodium hydroxide, 0.1N

Isotonic sodium sulphate 30 g/l of anhydrous salt

Diacetyl monoxime, 3% (w/v) in water, this solution is stable indefinitely if kept in the refrigerator and for a considerable time at room temperature.

N Phenylanthranilic acid, 0.005M. Phenylanthranilic acid (0.106 g) dissolved in water containing Na_2CO_3 (0.05 g), when dissolution is complete the solution is diluted to 100 ml.

Sulphuric acid 18N, made by mixing equal vols of concentrated acid and water.

Potassium persulphate, 1% (w/v). This is stable for 2 months in the refrigerator.

Urea standards Urea of B.P.C. purity, m.p. 130–132°, is adequate for the preparation of standards. The stock standard, 0.1% (w/v), will keep for a month or more in the refrigerator. From this the working standards are prepared at frequent intervals by suitable dilution. For visual

colorimetry three standards should be used containing respectively 3, 6 and 12 μg urea/ml., with the procedure for 0.1 ml samples these are equivalent to blood ureas of 30, 60 and 120 mg/100 ml. If a photoelectric colorimeter is used a single standard containing 5 μg urea/ml. is most suitable, this gives an extinction of c 0.40 with the Ilford spectrum green filter

Procedure for 0.1 ml samples

Into a centrifuge tube containing 7.9 ml. of isotonic Na_2SO_4 pipette 0.1 ml. of blood. Add 1 ml. of 2% ZnSO_4 followed by 1 ml. of 0.1N NaOH , mix and filter. Sufficient filtrate is obtained to enable the determination to be performed in triplicate if desired. Pipette 2 ml. of the filtrate into a test tube. Into similar tubes pipette 2 ml. of the standards, and, if a photoelectric colorimeter is used, prepare a blank using 2 ml. of water. To each tube add 0.25 ml. of 3% diacetyl monoxime and 0.25 ml. of 0.005M phenyl anthranilic acid followed by 4 ml. of 18N H_2SO_4 . Mix thoroughly and place in a boiling water bath for 10 min. Remove from the bath and without cooling add at once 0.25 ml. of 1% potassium persulphate. Mix thoroughly and allow the tubes to stand for 15 min. for the colour to develop, when a visual colorimeter is used 10 min. suffices. Compare using the Ilford spectrum green filter. For a blood urea over 150 mg/100 ml. it is best to repeat the determination using 1 ml. of the filtrate, although a fairly reliable result can be obtained by diluting with water before comparing.

Procedure for 0.02 ml samples

Into a centrifuge tube containing 2.5 ml. of isotonic Na_2SO_4 pipette 0.02 ml. of blood, add 0.2 ml. of 2% ZnSO_4 followed by 0.2 ml. of 0.1N NaOH . Mix and centrifuge. Pipette a 2 ml. sample (equivalent to 0.0137 ml. of blood) into a clean tube, taking precautions to avoid contamination by any of the precipitate, and treat exactly as described for 0.1 ml samples. The same standards can be used, and the result is multiplied by 1.46 to allow for the extra dilution.

RESULTS

The method has been compared with the manometric hypobromite method of Van Slyke & Kugel (1933), and fairly good agreement has been obtained (Table 1). In 80% of cases the difference between the two methods was less than 3 mg/100 ml urea,

Table 1 *Comparison of the improved diacetyl reaction for urea with the Van Slyke manometric hypobromite method*

Range of differences (mg/100 ml)	Percentage of cases
- 10 to - 6	8.6
- 6 to - 4	2.9
- 4 to - 2	17.1
- 2 to + 2	54.4
+ 2 to + 4	5.7
+ 4 to + 6	8.6
+ 6 to + 10	2.9

but in 10% of cases errors of 8–10 mg/100 ml were obtained. In spite of this, these results compare favourably with other colorimetric urea methods in current use, and the method can be recommended for routine analysis where only small quantities of blood are available. It is not sufficiently accurate for urea clearance tests. It has the disadvantage that the final solution is strongly acid. Attempts to overcome this were unsuccessful since dilution with water causes precipitation of the colour complex, whilst dilution with ethanol causes the colour to fade. If acetone is used as diluent the colour develops in the absence of persulphate, but unfortunately only reaches its maximum value after 3 hr. standing.

SUMMARY

1. When the condensation of urea with diacetyl is carried out in the presence of an aromatic amine a red colour is produced, instead of the usual yellow. With diphenylamine derivatives an intense magenta colour is produced.

2. The reaction with *N*-phenylanthranilic acid has been studied in detail and a method for the determination of urea in blood has been derived.

3. The estimation can be performed with as little as 0.02 ml. of blood, and results sufficiently accurate for most clinical purposes are obtained.

I wish to thank Prof. N. F. MacLagan for advice and encouragement in the writing of this paper.

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The Chemical Conversion of Nicotinic Acid and Nicotinamide to Derivatives of *N*-Methyl-2-pyridone by Methylation and Oxidation

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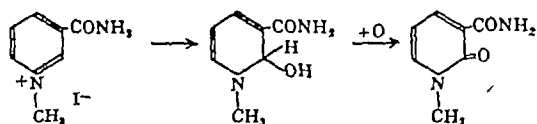
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The recent work of Knox (1946) and of Knox & Grossman (1946, 1947) has shown that *N* methyl nicotinamide chloride can be oxidized by an enzyme present in rabbit liver to the amide of *N*-methyl 2-pyridone 5 carboxylic acid. These workers have also reported the isolation of the amide of *N* methyl 2-pyridone 5 carboxylic acid from human urine after the ingestion of 0.6–0.9 g of nicotinamide per day. It is already well known that nicotinamide is methylated in the human body and that *N* methyl nicotinamide is normally excreted in human urine. The results of Knox & Grossman (1946, 1947) therefore indicate that part of the *N* methyl nicotinamide formed from nicotinamide in the human body may be oxidized by liver enzymes to *N* methyl 2-pyridone 5 carboxylic acid amide.

The amide of *N* methyl 2-pyridone 5 carboxylic acid has not previously been described, but the acid itself was prepared and described by von Pechmann & Welsh (1884), who synthesized it by two methods, viz. by the action of methylamine on the methyl ester of coumalic acid, with subsequent saponification, and by evaporating 2-pyridone 5 carboxylic acid to dryness with potassium hydroxide (2 mol) and methylating the salt with methyl iodide in methanol. Meyer (1905) prepared the acid by methylating 2-pyridone 5 carboxylic acid with methyl iodide and aqueous alkali.

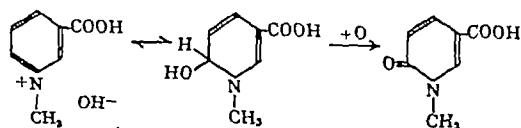
Neither the method of preparation from coumalic acid and methylamine, which involves the building up of the entire ring structure, nor the method of preparation by methylation of 2-pyridone 5 carboxylic acid, explains how the *N* methyl nicotinamide is converted into the pyridone derivatives *in vitro* or *in vivo*. It seemed, therefore, of interest to see if *N* methyl 2-pyridone 5 carboxylic acid amide can be formed from *N* methyl nicotinamide by the application of the method of Decker (1892, 1893) for the conversion of pyridinium compounds to 2-pyridones by means of alkaline ferricyanide. *N* Methyl nicotinamide was prepared by methylation of nicotinamide, and was oxidized with alkaline ferricyanide. By extraction of the reaction mixture with chloroform a compound of m.p. 216–

216.5°* was isolated, which had the same elementary composition as the amide of *N* methyl 2-pyridone-5 carboxylic acid, but which showed ultraviolet absorption properties entirely different from those reported by Knox & Grossman (1947), and which fluoresced strongly in ultraviolet light. In order to check the identity of this compound it was saponified. An acid was obtained with m.p. 183–183.5°. This acid was *N*-methyl 2-pyridone 3 carboxylic acid, for which Späth & Koller (1923) reported a melting point of 184°, and not *N* methyl-2-pyridone-5 carboxylic acid, which has a widely different melting point (237–238° according to von Pechmann & Welsh (1884), 238–239° according to Meyer (1905)). The following series of reactions therefore occurred:



The product of the oxidation of *N*-methyl nicotinamide was the amide of *N*-methyl-2-pyridone 3 carboxylic acid and not that of *N* methyl 2-pyridone 5 carboxylic acid.

In a search of the literature a patent (D.R.P. no. 522,060) of Rath (1932) was found in which it was stated that *N* methyl-2-pyridone 5 carboxylic acid may be prepared from nicotinic acid by methylation with methyl sulphate followed by oxidation. Nicotinic acid was therefore methylated by a method similar to that used by Winterstein & Weinhausen (1917), and the product was oxidized with alkaline ferricyanide. A good yield of *N* methyl-2-pyridone 5 carboxylic acid (m.p. 238–238.5°) was obtained from the reaction mixture. The following reactions occurred:

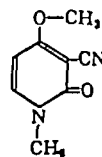


* Unless otherwise stated, the melting points given in this paper are uncorrected.

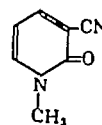
It seems that, *in vitro*, the methylation and oxidation of nicotinic acid yields *N*-methyl 2 pyridone 5 carboxylic acid, while the methylation and oxidation of nicotinamide gives the amide of *N*-methyl-2 pyridone 3 carboxylic acid. There is no evidence to suggest that mixtures of both isomers are formed in either of these reactions, since, if both amides were formed by the oxidation of *N*-methyl-nicotinamide, they would be extracted by chloroform* and would give, after saponification, a mixture of acids with an ill defined melting point, if both acids were formed by the methylation and oxidation of nicotinic acid, they would be precipitated by hydrochloric acid and would also give a mixture with an incorrect melting point. It was thought possible that the course of the reactions might be influenced by the nature of the methylating agent used. To test this point nicotinic acid was methylated with methyl iodide and the product oxidized, and nicotinamide was methylated with methyl sulphate and the *N*-methyl compound was oxidized. The same results were obtained as in the previous experiments, viz nicotinic acid gave *N*-methyl 2-pyridone 5 carboxylic acid, and nicotinamide gave *N*-methyl-2 pyridone-3-carboxylic acid amide. The methylating agent, therefore, had no influence on the course of either of the reactions.

amide by a different series of reactions in the animal body from that *in vitro*, or that *N*-methyl 2 pyridone 5-carboxylic acid is first formed from nicotinic acid and is subsequently converted to the amide.

The amide of *N*-methyl 2-pyridone-3 carboxylic acid, which is formed by the oxidation of *N* methyl nicotinamide *in vitro*, but not in the animal body, has a similar structure to two compounds of interest in plant biochemistry, viz ricinine and ricinidine. It is conceivable that plants may be capable of producing these substances from *N*-methylpyridinium compounds by oxidation.



Ricinine



Ricinidine

The amide of *N* methyl-2-pyridone-5 carboxylic acid was prepared by converting the acid to the acid chloride with thionyl chloride and by decomposing the acid chloride with aqueous ammonia, it was also prepared from the ethyl ester of the acid. The ultra violet absorption of the amide prepared by these methods (see Fig 1) was identical with that reported

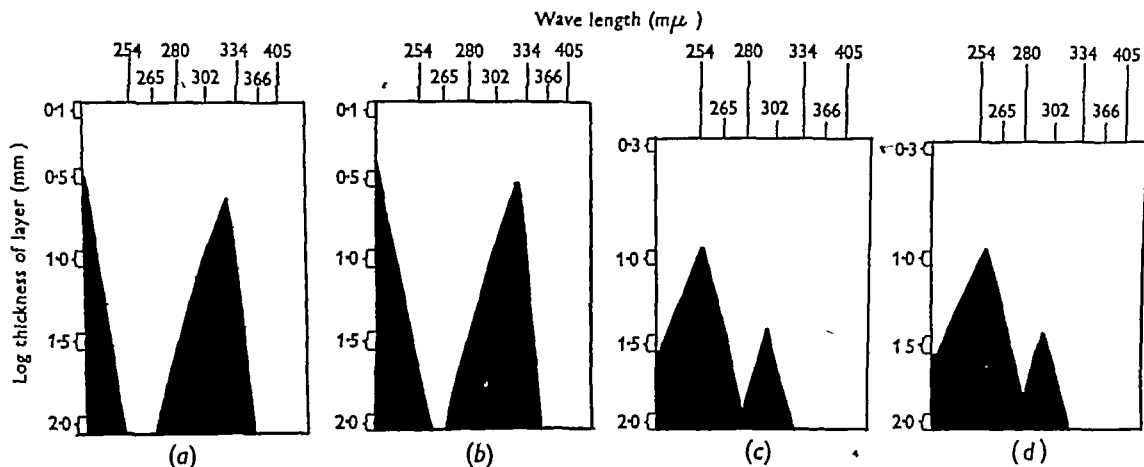


Fig 1 Approximate diagrammatic reproductions of ultraviolet absorption photographs (a) *N* Methyl 2 pyridone 3-carboxylic acid, 0.005 % in water (b) *N* Methyl 2 pyridone 3 carboxylic acid amide, 0.005 % in water (c) *N* Methyl 2-pyridone 5 carboxylic acid, 0.001 % in water (d) *N* Methyl 2 pyridone 5 carboxylic acid amide, 0.001 % in water

The results of the above experiments indicate either that the amide of *N*-methyl 2 pyridone 5 carboxylic acid is produced from *N* methylnicotin-

* See later sections for the preparation of the amide of *N* methyl 2-pyridone-5 carboxylic acid. Both the 2 3 and the 2 5 amides are slightly soluble in chloroform and are reasonably stable in the alkaline ferrieyanide solution. Neither of the isomeric acids could be isolated from the mother liquor after extraction with chloroform.

by Knox & Grossman (1947). Its elementary composition agreed closely with the theoretical values. The melting points of four samples prepared by different methods (201–204°, 204–206.5°, 203–205°, 202–203° (205.5–206.5° corrected)) were definitely lower than that of 212–214°, which was found by Knox & Grossman (1947) for *N* methyl 2 pyridone 5 carboxylic acid amide prepared enzymically from *N*-methylnicotinamide chloride. Further

efforts to test the purity of our specimens of the amide showed that the acid (m p 236–237°) could be recovered by saponification with 2.5N sodium hydroxide, and that no change in the melting point resulted after repeated recrystallizations from various solvents, after extraction with chloroform or ether, or after previous heating of the substance to its melting point.

It is interesting to observe (Fig. 1) that there is a wide difference in ultraviolet absorption between the amide of *N*-methyl-2-pyridone 3-carboxylic acid and that of *N*-methyl 2-pyridone 5-carboxylic acid. The former compound shows strong blue fluorescence in ultraviolet light, the latter has no fluorescence. Knox & Grossman (1947) stated that *N*-methyl-2-pyridone 5-carboxylic acid amide fluoresces slightly under the conditions used in the estimation of *N*-methylnicotinamide in urine, but their preparations, which were not white in colour, may have contained traces of impurities.

The present results suggest that, in the estimation of aneurin in urine by the thiochrome method, the alkaline ferricyanide used to oxidize aneurin to thiochrome may also convert a part, at least, of the *N*-methylnicotinamide present to the fluorescent *N*-methyl 2-pyridone 3-carboxylic acid amide, which would then be extracted by isobutanol together with the thiochrome. The error, which Najjar & Ketron (1944) found to be introduced into the estimation of aneurin in urine by the presence of *N*-methylnicotinamide, may be attributable to a difference in the intensity of fluorescence of *N*-methyl 2-pyridone 3-carboxylic acid amide compared with that of the product of the reaction of alkali alone on *N*-methyl nicotinamide, and/or to partial destruction of the latter substance by alkaline ferricyanide and incomplete formation of the former. Najjar & Ketron (1944) suggested that *N*-methylnicotinamide was converted to a pyridone by alkali and ferricyanide, but they did not isolate and identify the compound.

EXPERIMENTAL

N-Methylnicotinamide iodide

Nicotinamide (25 g) was dissolved in methanol (50 ml.) and the solution boiled gently under reflux for 8 hr with 43.7 g methyl iodide (1.5 mol.). After removal of methanol and of the excess of methyl iodide by filtration, *N*-methyl nicotinamide iodide remained as a pale yellow crystalline mass (54.3 g) of m p 203–204° (cf m p 204° reported by Karrer, Schwarzenbach, Benz & Solmssen, 1936).

N-Methyl 2-pyridone 3-carboxylic acid amide

N-Methylnicotinamide iodide (20 g) was dissolved in water (120 ml.) in a 1 l. flask. The flask was cooled in ice. To the contents were added slowly, with constant stirring, a solution of 53 g $K_3Fe(CN)_6$ in 170 ml. of water, and, simultaneously, a solution of 20 g KOH in 40 ml. water. The mixture was stirred for 1 hr. The precipitated $K_3Fe(CN)_6$

was filtered off and *N*-methyl 2-pyridone 3-carboxylic acid amide was isolated from the filtrate by extracting repeatedly with chloroform. After evaporation of the combined chloroform extracts to dryness, the pale brown residue (2 g) was purified by crystallization from methanol. The compound crystallized in the form of white needles with a faint greenish fluorescence, m p 216–216.5°. Späth & Koller (1923) reported a m p of 216° for this compound (Found: C, 55.6, H, 5.64, N, 18.4. Calc. for $C_7H_8O_2N_2$: C, 55.2, H, 5.26, N, 18.4%). Both the 2,3- and the 2,5-amides tend to leave a residue which contains N and C. Care must therefore be taken in the determination of N and C to ensure complete combustion.

N-Methyl 2-pyridone 3-carboxylic acid

N-Methyl 2-pyridone 3-carboxylic acid amide (0.5 g) was saponified by boiling gently under reflux for 3 hr with 2.5 ml of 2.5N NaOH. The solution was acidified with 2N HCl and the precipitated *N*-methyl 2-pyridone 3-carboxylic acid was filtered off. After crystallization from water the acid was obtained as thin, pure white needles of m p 183–183.5°. Späth & Koller (1923), who prepared this compound by another method, found m p 184°.

N-Methyl 2-pyridone 5-carboxylic acid

Nicotinic acid (82 g) was covered with methanol (200 ml.), and the mixture boiled gently under reflux with 90 g methyl sulphate. After about 0.25 hr the nicotinic acid dissolved. After heating for 2 hr the methanol and the excess of methyl sulphate were removed *in vacuo*. The viscous brown liquid which remained was transferred to a 3 l. flask with 200 ml. water. The flask was cooled in ice, and to the contents were added, drop by drop, with constant stirring, a solution of 440 g $K_3Fe(CN)_6$ in 750 ml. water, and, simultaneously, a solution of 194 g KOH in 500 ml. water. The resulting mixture was stirred for 1 hr and was filtered to remove precipitated $K_3Fe(CN)_6$. The filtrate was acidified with 6N HCl. The precipitated *N*-methyl 2-pyridone-5-carboxylic acid was filtered off and washed on the filter four times with cold water (yield 60 g). It was dissolved in hot water and the solution boiled with charcoal. After crystallization from water, white needles (30 g) were obtained with m p 238–238.5°. This melting point agrees well with the values of 237–238° reported by von Pechmann & Welsh (1884), and of 238–239° found by Meyer (1905).

N-Methyl-2-pyridone 5-carboxylic acid ethyl ester

N-Methyl 2-pyridone 5-carboxylic acid (10 g) was esterified by heating for 6 hr with absolute ethanol (100 ml.), through which a stream of dry HCl was bubbled continuously. The ethanol and HCl were removed by evaporation *in vacuo*. The dry residue was ground in a mortar with solid Na_2CO_3 and the ester (m p 72.5–74°) was extracted with benzene. It was purified by crystallization from a mixture of benzene (1 vol.) and ligroin (3 vol.), m p 72–73.5° (cf m p 74° reported by Rätz & Schiffmann, 1931).

N-methyl 2-pyridone 5-carboxylic acid amide

(a) *N*-Methyl 2-pyridone 5-carboxylic acid (10 g) was boiled gently under reflux for 1–2 hr with 50 ml. thionyl chloride. The excess of thionyl chloride was evaporated

in vacuo The greenish yellow crystalline residue was ground in a mortar, and was slowly added to 200 ml conc NH_3 , with cooling. The mixture was stirred continuously for 3 hr. *N*-Methyl 2-pyridone 5-carboxylic acid amide was precipitated as a white crystalline mass, which was filtered off (yield 5 g, m.p. 201–203.5°), and purified by crystallization from absolute ethanol (pure white needles of m.p. 201–204°) (Found C, 54.8, H, 5.32, N, 18.2, amide N, 9.0. Calc. for $\text{C}_7\text{H}_8\text{O}_2\text{N}_2$: C, 55.2, H, 5.26, N, 18.4, amide N, 9.2%).

(b) The same procedure was used as in (a), except that 22.5 g of *N*-methyl 2-pyridone 5-carboxylic acid and correspondingly greater amounts of the other reagents were taken, and the time of heating with thionyl chloride was somewhat shorter (1 hr). The yield of the crude product was 15 g. After two recrystallizations from absolute ethanol 8.5 g of white needles of m.p. 204–206.5° were obtained.

(c) The procedure was the same as in (a), except that the time of heating with thionyl chloride was reduced to 20 min. After crystallizing once from absolute ethanol, white needles of m.p. 202–203.5° were obtained. The product, however, contained a trace of an impurity, presumably unchanged acid, which melted at 233.5°. After treatment with a solution of NaHCO_3 and crystallization from absolute ethanol, no trace of the high melting impurity remained and the melting point was sharp (202–203°, uncorrected, 205.5–206.5°, corrected). After saponification for 7 hr with 2.5N NaOH , *N*-methyl 2-pyridone 5-carboxylic acid was recovered (m.p. 236–237°).

(d) *N*-Methyl 2-pyridone 5-carboxylic acid ethyl ester (5 g) was heated for 8 hr at 200° under a pressure of

63 kg/sq cm with 20 ml methanol saturated with NH_3 . After removal of methanol and NH_3 by evaporation on a steam bath, the residue was extracted once with benzene to remove unchanged ester. The amide of *N*-methyl 2-pyridone 5-carboxylic acid was isolated from the benzene insoluble residue by crystallization from absolute ethanol. It was purified by a second crystallization from absolute ethanol, almost white crystals of m.p. 203–205° were obtained.

SUMMARY

1. Methods are described for the preparation of the amide of *N*-methyl-2-pyridone 3-carboxylic acid by the methylation and oxidation of nicotinamide, and for the preparation of *N*-methyl-2-pyridone 5-carboxylic acid by the methylation and oxidation of nicotinic acid. Methods are also given for the conversion of the former compound to the corresponding acid, and of the latter compound to the corresponding amide.

2. The properties of the two isomeric acids and amides are compared. The course of the reactions which occur in the formation of these compounds *in vitro* is discussed in relation to the biological formation of the amide of *N*-methyl-2-pyridone 5-carboxylic acid from nicotinamide in the human body.

We are indebted to Dr E. Merkel for the ultraviolet absorption photographs and to Dr O. Wollenberg for the elementary analyses.

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Absorption of 3-Methylglucose from the Small Intestine of the Rat and the Cat

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On the basis of the phosphorylation theory (Verzár & McDougall, 1936) certain sugars are absorbed from the intestine by an active process, one stage of which involves the phosphorylation of the sugars. Sugars so absorbed are thought to leave the gut more rapidly than those depending only on simple diffusion, and it is customary to judge whether or not a sugar is actively absorbed by comparing its

rate of absorption with that of glucose. On this criterion, galactose and glucose are classed as being actively absorbed whilst the pentoses, such as xylose, are thought to enter by simple diffusion only. In the course of work on the synthetic 3-methylglucose, it became of interest to determine whether it was actively absorbed from the intestine. Two approaches were made: first, the rate of absorption from the

rat's small intestine was compared with that of glucose, secondly, the power of the gut to absorb the sugar against a concentration gradient was investigated in the cat. Both lines of inquiry indicated the existence of an active mechanism for the absorption of 3-methylglucose.

METHODS

Experiments on rats

In individual rats, under nembutal anaesthesia, two adjacent loops of the small intestine were isolated with ligatures, isotonic solutions of glucose and the sugar to be examined were placed in the separate loops and, after a given time, the contents were washed out and their reducing values determined by the Hagedorn & Jensen (1923) technique. From rat to rat the relative positions of the two solutions, proximal or distal to the stomach, were alternated in order to discount variations in the absorptive rate due to their position in the gut. The technique closely followed that described by Davidson & Garry (1941).

Experiments on cats

The general principle of these experiments consisted in maintaining a concentration of 3-methylglucose in the blood higher than that in the gut. If the sugar enters the blood only by simple diffusion, the absorption must take place as a result of the previous absorption of water to such an extent that the concentration of the sugar in the gut is greater than that in the blood. After a given time, then, any loss of sugar from the gut would have to be accompanied by a concentration in the gut equal to, or greater than, that in the blood, if simple diffusion were the only factor. The absorption of water could be by a specific process, or it could simply result from the colloid osmotic pressure of the plasma.

Thus an estimation of the amount of 3-methylglucose absorbed from an isolated loop, combined with a determination of the concentration of 3-methylglucose in the blood during the process of absorption, and in the gut fluid at the end of the absorptive period, should provide unequivocal evidence as to whether the sugar is actively absorbed, since a loss of sugar from the intestine, associated with a lower intestinal concentration than that in the blood, could not arise by a simple diffusion process. To reduce colloid osmotic influences to a minimum, the sugar was dissolved in the cat's own plasma. To ensure that the concentration in the blood would be the higher, the animal was given an intravenous injection of the sugar after the renal arteries had been tied. A large sample of blood was withdrawn, the plasma separated and 5 ml inserted into an isolated loop, meanwhile, successive intravenous injections of the sugar were administered during the course of the experiment in amounts designed to maintain the blood level definitely

higher than that in the gut. For control purposes a second loop was isolated and the animal's plasma, withdrawn before the injection of 3-methylglucose, inserted, and the absorption of glucose estimated.

The cats were cleared of intestinal worms with a dose of 10 mg of arecoline hydrobromide in milk. The renal arteries were tied under nembutal anaesthesia. The femoral vein of one leg and the artery of the other were cannulated, and 10 ml of blood withdrawn to provide the plasma for the control glucose absorption. A volume of 18 ml of the 6% (w/v) 3-methylglucose in 6% gum acacia was injected and, after time had been allowed for equilibration, 10 ml of blood were withdrawn and the plasma separated. The abdomen was then opened and the warm plasmas were inserted into adjoining isolated loops of the small intestine, the technique of Davidson & Garry (1940) being followed. During the whole of the procedure, the injection of 3-methylglucose was continued at the rate of about 2 ml every 5 min. After a given interval the loops were removed and emptied, 0.1 ml samples of the contents were taken for reducing value estimations and the remainder, together with the washings of the gut, was made up to 25 ml for Zeisel methoxyl determinations (Elek, 1939) of 3-methylglucose. Methoxyl and reducing values were also estimated on the blood samples.

In order to determine more accurately the changes in the sugar concentration of the gut fluid during the absorptive period, a second series of experiments was conducted in which a single loop was well filled with the plasma containing 3-methylglucose. The loop was emptied after a short absorption period, when at least 2 ml of fluid remained unabsorbed. This permitted an accurate duplicate determination of the actual concentration of 3-methylglucose in the fluid. All estimations in this case were done by removing the glucose by yeast fermentation and estimating the non-fermentable 3-methylglucose by the Hagedorn & Jensen technique. The fermentation technique followed closely that described by Young (1938) and Somogyi (1927).

RESULTS

Experiments on rats

Sixteen rats were treated and the results calculated as the absorption in mg/g of gut/hr. The results are shown in Table 1. If the rate of absorption of glucose be taken as 100 then that of 3-methylglucose is 84.

It was found that if the absorption took place in the proximal part of the intestine there was no difference between the caudal and cranial loops, whereas if more of the intestine was used and the more distal portion included there was a marked difference in the rates of absorption from the two loops, that from the caudal being appreciably less than that from the cranial loop. These differences, however, were eliminated in computing the results, since in eight rats the one sugar was in the caudal loop and in the other eight rats the position was reversed.

Table 1 *Absorption of sugars from rat intestine*

(Absorption rates as mg sugar/g of gut/hr)		
	3 Methylglucose	Glucose
Mean value	32.2	38.3
Standard error	2.06	1.00
Extreme ranges		
Highest	44.7	47.0
Lowest	21.1	24.2
Difference = 15.9%		

Experiments on cats

In the first series of experiments it was found that in each of three cats, in which the concentration of 3-methylglucose in the blood was kept above that in the gut, appreciable absorption did take place. The results are summarized in Tables 2 and 3. It will be seen from these tables that (a) the percentage absorption is comparable with that of glucose, and (b) the initial concentration of 3-methylglucose in the intestine was considerably less than that in the blood. The results giving the final concentration of 3-methylglucose were inconclusive in this series and probably not very accurate, since very little plasma remained in the intestine after the absorption period.

Table 2 *Percentage absorption of sugars in loops of cats' intestine*

Cat	3 Methylglucose	Glucose
I	30	40
II	38	35
V	50	60

Table 3 *Concentration of 3-methylglucose during absorption in the cat*

(Concentration in mg/100 ml. plasma)

	Cat		
	I	II	V
Av. concentration of 3-methylglucose in blood during absorption	690	400	230
Initial concentration of 3-methylglucose in intestine	470	250	190

Table 4 *Concentration of 3-methylglucose during absorption in the cat*

Concentration of 3-methylglucose (mg/100 ml. plasma)	Cat		
	VI	VII	VIII
In intestine Initially	219	249	231
After absorption	189	206	178
In blood Before absorption	261	240	229
After absorption	342	263	238

In the second series of experiments (Table 4) it was found that, in each of three cats in which the concentration of 3-methylglucose in the blood was

maintained at or above the concentration in the intestine, an appreciable lowering of the concentration of 3-methylglucose took place.

DISCUSSION

Amongst others Cori (1925*a, b*) and Wilbrandt & Laszt (1933) have shown that different sugars are absorbed at different rates from the gut. In general, the hexoses are absorbed more rapidly than the pentoses. It has been suggested that the difference in the rates between these two groups of sugars is due to the fact that the hexoses (glucose and galactose) are actively absorbed, whereas the pentoses are merely absorbed by simple diffusion. Verzář & McDougall (1936) suggested that the mechanism of active absorption may be phosphorylation, a theory which is supported by the fact that glucose and galactose are phosphorylated *in vivo* whereas the pentoses are not.

Table 5 *Absorption rates of various sugars from rat intestine*

Galactose	115	Fructose	44
Glucose	100	Pentoses	30
3 Methylglucose	84		

Table 5 shows a comparison between the rates of absorption of various sugars from the rat's intestine (Wilbrandt & Laszt, 1933) to which our results with 3-methylglucose have been added. It will be seen from this that 3-methylglucose, on the above basis, would be expected to be actively absorbed in the rat. The fact that the sugars were apparently absorbed more slowly the more distal the loop from the stomach, coupled with the evidence of Davidson & Garry (1941) that there was no difference in the rates of absorption of the pentoses in the caudal and cranial loops of the rat's intestine, suggests that the efficiency of the 'active' absorption mechanism decreases along the length of the small intestine.

In the experiments in the cat there can be little doubt that absorption did take place against a concentration gradient. It has also been shown that the concentration of sugar in the gut loop decreased during absorption so that there can be no question that absorption took place by preferential absorption of water which would upset the gradient. Bárány & Sperber (1939) have also shown that glucose can be absorbed against a concentration gradient, our results confirm this. We must, therefore, conclude that the 3-methylglucose was actively absorbed in the cat.

Previous results have shown that 3-methylglucose is not glycogenic in the rat (Campbell & Young, 1948), so that from this standpoint it would not be expected to be actively absorbed. Therefore, the

fact that it is, suggests that phosphorylation is not the mechanism of active absorption in this case. These results confirm those of Davidson & Garry (1940) in which they found that D xylose is absorbed as rapidly as glucose from the caudal region of the small intestine of the cat. Shapiro (1947) has suggested from results with phlorrhizin and rat kidney extracts that, although phosphorylation may play a part in the absorption of sugars, it is probably far from being the only mechanism, and this is also true of the small intestines of the rat and the cat.

SUMMARY -

1 In rats the rate of absorption of 3 methylglucose has been found to be about 16 % slower than that of glucose.

2 In cats 3 methylglucose has been found to be absorbed against a concentration gradient.

3 Since 3 methylglucose is not glycogenic in the rat, it is suggested that phosphorylation is not the only mechanism of active absorption either in the rat or the cat.

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Determination of Traces of Iron and Copper in Culture Media Prepared by Enzymic Digestion of Muscle Protein

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During the course of experimental work on the effect of trace metals on the toxin production of *Corynebacterium diphtheriae*, it became apparent that a very sensitive method for the determination of iron and copper was necessary. As a temporary measure, a method for iron was evolved, based on Mueller's (1938) media deferration method, consisting of adding calcium phosphate to the sample, precipitating with ammonia, and treating the precipitate with o-phenanthroline to develop a colour with the ferric phosphate adsorbed on the calcium phosphate. This method gave reliable and reproducible values for inorganic iron, but gives no indication of the amount of bound iron present. The problem thus resolved itself into two main steps: (a) incineration of the sample, and (b) the determination of iron and copper in the ash.

Dry ashing methods, particularly that due to Klump (1934), were investigated and rejected because of the significant amounts of iron and copper extracted from the ashing vessels. Silica, porcelain and pyrex crucibles all gave trouble, a fact noted by Braun & Scheffer (1940), but platinum was not tried since Bailey & McHargue (1945)

stated that it gave a colour with the copper reagent similar to that given by copper itself. Wet ashing with H_2SO_4 , HClO_4 and HNO_3 in pyrex flasks was then tried, but even after diminishing the large acid blank by redistillation of the acids, results were still erratic and high. It was not until the flasks were specially cleaned that consistent results were obtained. Wet ashing was a lengthy procedure until Smith's (1946) method was investigated, in which digestion was greatly accelerated by the use of vanadium as a catalyst. This method gave no trouble provided that the rate of heating was controlled to prevent spontaneous ignition.

Of a number of colorimetric reagents available for the determination of small quantities of iron, $\alpha\alpha'$ -dipyridyl and o-phenanthroline appeared to be the most satisfactory from the point of view of sensitivity, stability of colour and freedom from interference, and a number of methods employing both dipyridyl (Hill, 1930; Dyer & MacFarlane, 1938; Jackson, 1938; Schulek & Floderer, 1939; Thorpe, 1941; Koenig & Johnson, 1942; Moss & Mellon, 1942; Norlin, 1943; Kitzes, Elvehjem & Schuette, 1944) and phenanthroline (Saywell & Cunningham, 1937; Fortune & Mellon, 1938; Hummel & Willard, 1938; Schaefer, 1940; Mehlig & Hulett, 1942; Borei, 1943; Bandemer & Schaefer, 1944; Benne & Snyder, 1944) is described in the literature. The thiocyanate complex was rejected in view of its in

stability and sensitivity to variation of pH, etc (Woods & Mellon, 1941, Benne & Snyder, 1944) Phenanthroline is about one and a half times as sensitive as dipyrndyl, otherwise there is little to choose between the two reagents. They both form coloured complexes with ferrous iron and give practically no colour with ferric iron, so an efficient reducing agent must be used. A number of different reducing agents has been employed, of which the most satisfactory appear to be hydroxylamine hydrochloride (Fortune & Mellon, 1938, Moss & Mellon, 1942) and *p* hydroxyphenyl glycine (Dyer & MacFarlane, 1938). Sodium hyposulphite ($\text{Na}_2\text{S}_2\text{O}_4$), though efficient, is somewhat unstable, and grades obtainable contained considerable quantities of iron.

The estimation of iron in the ash was relatively simple when phosphate was absent, but in the presence of relatively large amounts (up to 10,000 l) of this ion it became difficult owing to the formation of ferric pyrophosphate during ashing. Several methods have been described to overcome this difficulty, of which the methods of Schulek & Floderer (1939), as described by Moss & Mellon (1942), and Jackson (1938) appeared to be most promising. Schulek & Floderer's (1939) phosphoric acid procedure was found to give fairly good results with tryptic digests but not with papain digests, a satisfactory explanation for this was not found. Jackson's (1938) method of precipitating the iron as sulphide was not satisfactory since filters would not hold the precipitate. Adsorption of the precipitate on $\text{Ca}_3(\text{PO}_4)_2$, and PbS , although it gave better results, was not entirely satisfactory. Attempts to prevent the formation of pyrophosphate by omitting H_2SO_4 from the digestion mixture were not successful since the presence of HClO_4 in the residue caused the precipitation of what was presumably ferrous phenanthroline perchlorate, for which Smith & Richter (1944) give a solubility of 0.00103 mol/l. Destruction of the excess HClO_4 with hydrazine, hydroxylamine and oxalic acid was attempted but was unsuccessful owing to the large quantities of reducing agent required. Other types of reducing agents could not be used since the decomposition products had to be volatile to limit the concentration of solids in the final solution. Hydrated silica also caused trouble by adsorbing some of the coloured iron complex on standing.

The problem was finally solved by heating the ash solution to boiling with phenanthroline and hydroxylamine, when it was found that maximum colour development was obtained in less than 30 sec. As a matter of interest, this phenomenon was more fully investigated and it was found that a similar effect was obtained in the presence of other complexes, 20 μg of Fe^{+++} were added to c. 0.1 g of the complexing ion (as the Na or K salt) in 5 ml of water, followed by phenanthroline, hydroxylamine and acetate buffer. If in 5 min the solution had not attained the same colour as a standard, it was heated to boiling. If the colours still did not match, a tiny crystal of sodium sulphide was added and the solution reheated. The results obtained are set out in Table 1.

In addition to the methods noted above for the determination of copper (Braun & Scheffer, 1940, Bailey & McHargue, 1945), a trichloroacetic acid

Table 1 *Colour development in presence of various complexing ions*

(0.1 g Na or K salt of complex, 20 μg Fe^{+++} , 0.25 ml 10% (w/v) hydroxylamine hydrochloride, 0.5 ml 0.25% (w/v) o-phenanthroline, 0.5 ml 1.7 M acetate buffer, diluted to 10 ml with distilled water)

Complexing ion	Maximal colour development
Benzoate	AH
Borogluconate	I
Citrate	AH
Ferricyanide*	AHS
Ferrocyanide*	AHS
Fluoride	AHS
Formate	I
Gluconate	AH
Glutamate	I
Glycerophosphate	AH
Glycollate	AH
Lactate	AH
Malate	AH
Maleate	AH
Mandelate	AH
Metaphosphate	AH
Orthophosphate	AH
Oxalate	AHS
Palmitate	Red ppt
Phenylacetate	AH
Phthalate	I
Pyrogallate	I
Pyrophosphate	AH
Pyruvate	Incomplete
Salicylate	AH
Stearate	I
Succinate	AH
Sulphosalicylate	AH
Tannate	Pale blue ppt
Tartrate	AH
Thiocyanate	AH
Thiodiglycollate	AH
Thiosulphate	I
Trichloroacetate	I

Key I=immediate, AH=after heating, AHS=after heating with sulphide, * total iron liberated.

extraction method using diethyldithiocarbamate (Tompsett, 1934a, Cartwright, Jones & Wintrobe, 1945) and Eden & Green's (1940) method using amyl alcohol extraction, were investigated. Tompsett's (1934a) method proved unsatisfactory, but Eden & Green's method gave excellent results.

The determination of copper in the ash presented no difficulties since the only interfering ion likely to be encountered was iron and that was firmly bound by the phenanthroline. The use of citrate as in Eden & Green's method was continued, however, since it held calcium phosphate in solution when the solution was made alkaline with ammonia (Tompsett, 1934b). Originally, total iron and copper were determined on separate samples, but in the light of the methods of Parker & Griffin (1939) and Gerber, Claassen & Boruff (1942), both elements were determined in the same sample.

METHOD

Apparatus and anti-contamination precautions

Great care had to be taken to exclude every chance of contamination by dust-borne and other extraneous iron at all stages of the procedure. Water, redistilled over AgNO_3 from a pyrex all glass still, was used and reagents were specially purified where necessary. It was found advisable to have a dust-proof cabinet (e.g. an old balance case) where tubes, flasks, reagents, etc. could be left free from contamination at any stage of the procedure.

Pyrex narrow necked conical flasks (250 ml.) were used for the incineration, and 10 ml graduated centrifuge tubes for the solutions after the development of the iron colour. The copper colour was developed in 40 ml pyrex glass stoppered tubes. When not undergoing heating, the flasks were covered with 50 ml. pyrex beakers. The flasks were specially cleaned to prevent contamination from the glass. When new, they were treated with boiling conc. H_2SO_4 for c. 30 min., and then with boiling aqua regia. Before use, they were treated each time with boiling 20% (w/v) NaOH to dissolve deposited silica (which appeared to cause erratic results) and then with boiling aqua regia. After acid treatment, the flasks were rinsed successively in tap, distilled and redistilled water and then inverted on a sheet of clean glass and allowed to drain. Tubes and pipettes were cleaned by immersing in chromic nitric acid cleaning mixture (2.5% (w/v) CrO_3 in conc. HNO_3) overnight and rinsing as above. (Chromic nitric acid mixture was found preferable to the conventional chromic sulphuric acid mixture. When the oxidizing properties of the acid were spent, the HNO_3 was recovered by distillation.)

For the incineration stage, a heater to accommodate six flasks was constructed from two 1 kW electric fire elements placed end to end and mounted on a suitable fire proof base. The elements were wired in parallel and rate of heating was controlled by a 'Variac' transformer. The elements were surrounded by a 'fence' of 'Polite' which projected to a height of approximately 3 in. above their surface. Along one side were cut six slots into which the flask necks were placed when tilting was necessary.

To prevent contamination by dust while the flasks were on the heater, a stream of completely dust-free air was blown into them. The filter system was of the utmost importance and after experiments the following train was found to be the most efficient: main air supply, 5 lb/sq in., glass wool column, P 16 'Aerox' filter, P 27 'Aerox' filter in water, H_2SO_4 wash bottle, trap. The system was connected to a manifold with six outlets, these being connected by rubber tubing to pieces of pyrex glass tubing bent so that they could be hooked on to the neck of the flask, with the outlet about 2 in. from the bottom.

Reagents

Sulphuric acid A.R. grade acid was distilled *in vacuo* over AgNO_3 to prevent volatilization of traces of FeCl_3 .

Nitric acid A.R. grade acid was purified as above.

Perchloric acid The 60% (w/v) acid was purified as above and collected as the constant boiling acid of 72% (w/v) strength.

Ammonium vanadate (0.25%) This (0.25 g) was dissolved in 75 ml of redistilled water with heating, cooled, a few drops of conc. NH_4OH solution added and the solution

diluted to 100 ml. The solution at first assumed a yellowish tint which soon faded.

Digestion mixture. The acids purified as above were mixed in the proportions by volume H_2SO_4 (1), HClO_4 (1.5), HNO_3 (7.5). Vanadate solution (2.5 ml) was added to 1 l of this mixture, giving a concentration of c. 5 $\mu\text{g}/\text{ml}$. The mixture was prepared immediately before use since it tended to extract iron from glass on standing.

Phenolphthalein (0.01%) The indicator (0.01 g) was dissolved in 50 ml of ethanol and the solution diluted to 100 ml with redistilled water with constant stirring. It was filtered (Whatman no. 44 paper) before use.

Ammonium hydroxide (sp. gr. 0.880) A.R. grade material was satisfactory after filtration through a Whatman no. 44 paper.

Hydrochloric acid (2N) A.R. grade acid was purified by distillation. The constant boiling mixture obtained (20.22% at 760 mm) was diluted to 2N (360 ml. diluted to 1 l.).

Hydroxylamine hydrochloride (10%) The hydrochloride (20 g) was dissolved in 100 ml. distilled water together with 1 g of $\text{Ca}_3(\text{PO}_4)_2$. The solution was adjusted to pH 8 with NH_4OH solution, warmed to flocculate the precipitate, then filtered through a Whatman no. 44 paper. The solution was then adjusted back to pH 2.5 with purified HCl and diluted to 200 ml.

o-Phenanthroline (0.25%) This was made up in 0.1N HCl . **Acetate buffer**, pH 4.6 Glacial acetic acid (50 ml.) was slowly neutralized with 10N NaOH (phenolphthalein indicator), the flask being well cooled with running water during the reaction. A further 50 ml. of glacial acetic acid were added and the solution diluted to 500 ml. The reagent was purified by the extraction method of Waring & Werkman (1943) as follows. To the solution in a pyrex separating funnel (with an ungreased tap) was added a solution of 10–20 mg of 8-hydroxyquinoline in 5 ml of redistilled metal free chloroform, and the mixture shaken vigorously for 1 min., then rotated for 30 sec. to cause the droplets to coalesce and sink. The chloroform was drawn off and the procedure repeated until this layer had only a slight yellow tint, two to three times usually being sufficient. Finally, the solution was washed with 10 ml. portions of chloroform until the chloroform extract caused no change in the colour of two to three drops of 20% ferric alum solution after vigorous shaking. The purified solution was then warmed to expel excess chloroform and finally filtered through a Whatman no. 44 paper.

Ammonium citrate (50% w/v) The reagent was purified by the extraction procedure described above for the acetate buffer.

Sodium diethylthiocarbamate (2%) This was dissolved in redistilled water with gentle heat, and filtered through a Whatman no. 44 paper each time before use to remove deposited sulphur, etc.

isoAmyl alcohol A.R. grade primary isoamyl alcohol could be used after one distillation, but commercial 'amyl alcohol' could only be used after purification by two to three distillations. It was readily recovered after use by drying over CaO followed by distillation.

Standard iron solution A solution containing 100 μg $\text{Fe}^{+++}/\text{ml}$ was prepared from A.R. $\text{NH}_4\text{Fe}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ (0.8636 g/l. 0.1N HCl).

Standard copper solution This solution contained 100 μg Cu^{++}/ml (0.3928 g of dry, recrystallized A.R. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ /l. 0.1N HCl).

The standards were diluted to give a combined working standard containing 20 μg Fe^{+++} and Cu^{++} /ml. All normalities referred to are approximate.

Procedure

The samples containing 2–20 μg of iron and copper were pipetted into clean flasks followed by 10 ml of digestion mixture. The flasks were placed on the heater and the air tubes inserted in the necks. A gentle stream of air was passed through, and the heating carefully controlled until the initial frothing period had passed (usually 3–5 min after boiling commenced), and the heat was then turned full on. When nitrogen peroxide fumes were evolved the flasks were removed from the heater and the air tubes removed. The heat was reduced to half, and after a few minutes the flasks were returned to the heater in the tilted position. The incineration was allowed to proceed until the solution was clear and the vigorous bubbling had ceased, then the flasks were righted, the air tubes reinserted and the heat turned to full until all the excess acid had been evaporated and a dry, white or pale yellow residue was obtained. The flasks were then removed from the heater, covered with beakers and allowed to cool.

The ash was dissolved in 3 ml of redistilled water, two drops of phenolphthalein added and the solution neutralized with concentrated ammonia solution. Normally, not more than one to five drops were necessary. The colour was discharged and precipitated calcium phosphate redissolved by the drop wise addition of 2N-hydrochloric acid. To prevent undue exposure of the flask contents, the iron colour developing reagents were mixed in the following proportions: hydroxylamine (1 vol.), *o* phenanthroline (2 vol.), acetate buffer (2 vol.), and 1.25 ml of this mixture were added to each flask. The flasks were then heated to boiling and the sides washed down by swirling the contents. Maximal colour was obtained in less than 30 sec. The solutions were then transferred to 10 ml graduated centrifuge tubes with 5 ml of redistilled water and the solutions diluted to 10 ml. If cloudy, the solutions were centrifuged or allowed to stand overnight in a dust-free cupboard.

The measurement of colour was carried out with a photoelectric absorptiometer against a water blank, using two Ilford filters 603 and 604 (spectrum blue green and spectrum green) together. The peak transmission of this filter combination corresponded closely with the maximal absorption of the ferrous

Table 2 Recoveries of iron and copper

Medium no	Fe) Cu) added (μg)	Iron			Copper		
		Found (μg)	Recovered (μg)	Recovery (%)	Found (μg)	Recovered (μg)	Recovery (%)
Controls	5.0	4.8	4.8	96	4.8	4.8	96
	10.0	9.6	9.6	96	9.8	9.8	98
	10.0	9.8	9.8	98	10.1	10.1	101
	10.0	9.7	9.7	97	9.8	9.8	98
BP 334*	0	2.6	—	—	22.5	—	—
	5.0	7.6	5.0	100	27.6	5.1	102
	10.0	12.2	9.6	96	32.4	9.8	98
BP 335	0	2.0	—	—	14.5	—	—
	5.0	7.1	5.1	102	19.5	5.0	100
	10.0	11.9	9.9	99	23.9	9.4	94
BP 336	0	3.1	—	—	20.5	—	—
	5.0	8.0	4.9	98	25.5	5.0	100
	10.0	12.7	9.6	96	30.2	9.7	97
BP 336A	0	3.6	—	—	8.2	—	—
	5.0	8.7	5.1	102	13.1	4.9	98
	10.0	13.3	9.7	97	18.4	10.2	102
BP 337	0	2.3	—	—	11.9	—	—
	5.0	7.2	4.9	98	17.1	5.2	104
BP 337A	0	2.3	—	—	5.8	—	—
	5.0	7.3	5.0	100	10.6	4.8	96
BP 339	0	4.0	—	—	5.4	—	—
	5.0	8.9	4.9	98	10.2	4.8	96
BP 339A	0	3.1	—	—	7.2	—	—
	5.0	7.9	4.8	96	12.4	5.2	104
		Mean		98	Mean		99
		Mean deviation		± 1.5	Mean		± 2.5

* BP=papain digest of beef muscle

phenanthroline complex at 508 m μ . Using 2 cm cells, the working range was 0–20 μ g Fe, but could be extended to 40 μ g with 1 cm cells and to 80 μ g with 0.5 cm cells. Beer's law was obeyed and the molar extinction coefficient obtained under these conditions was 11,150.

After reading, the coloured solutions were quantitatively transferred to the glass stoppered tubes using 5 ml of wash water. The following reagents were then added with mixing to these solutions in the order given: ammonium citrate (1 ml), concentrated ammonium hydroxide (1 ml) and diethyldithiocarbamate (0.25 ml). Exactly 10 ml of amyl alcohol were then added and the tubes shaken for not less than 30 sec in order to extract the copper complex completely. This colour was read after 15 min under the same conditions as for iron, but using two Ilford 601 (spectrum violet) filters and an amyl alcohol blank. The colour obeyed Beer's law giving a molar extinction coefficient of 11,650. The ranges of working could be extended to 80 μ g as with iron.

The transmission values obtained for both colours were converted to concentration of the respective metal from a standard curve which was prepared by taking a set of standards through the whole process, excluding ashing. A blank on all the reagents was carried out with each set of determinations.

RESULTS

Table 2 gives an indication of the accuracy of the method.

SUMMARY

1. A colorimetric method is described for the determination of the iron and copper (0–20 μ g) of meat digests in the same sample. Organic matter is destroyed by rapid wet ashing with a mixture of sulphuric, perchloric and nitric acids, using vanadium as a catalyst. Special precautions against dust and reagent contamination are necessary.

2. A novel feature is the decomposition of the ferric pyrophosphate complex by heating the ash solution with phenanthroline and a reducing agent, maximum colour, being developed in less than 30 sec.

3. Copper is determined as the diethyldithiocarbamate complex, after extraction with isoamyl alcohol.

4. Mean recoveries obtained are 98% (mean deviation $\pm 1.5\%$) for iron and 99% (mean deviation $\pm 2.5\%$) for copper.

I am indebted to Dr C. G. Pope for his helpful advice during the course of the work and the preparation of the manuscript.

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Synthesis of a Sulphur-containing Analogue of Thyroxine

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(Received 5 April 1948)

It is generally accepted that the obvious physiological effects of the thyroid hormone, as manifested by its influence on the metabolic rate, are brought about by the peripheral action of the hormone on the tissues. Although it is still not completely certain that the circulating hormone is free thyroxine itself the balance of evidence points in this direction (Harington, 1944), and this supposition is strongly supported by the recent work of Taurog & Chaikoff (1947).

In addition to its effect in stimulating the metabolism of tissues, thyroxine has an action on the anterior pituitary gland in regulating the output of thyrotrophic hormone by the latter organ. It is this effect which is responsible for the hypertrophy of the thyroid gland that results from treatment with one of the antithyroid drugs of the thiouracil type, under the action of these drugs thyroxine synthesis in the thyroid gland is blocked, with resulting diminution of the concentration of circulating thyroid hormone in the body, the response of the anterior pituitary gland to this situation is to release more thyrotrophic hormone, which in turn produces overgrowth of the thyroid gland. Conversely, if circulating thyroid hormone is increased by exogenous administration, thyrotrophic hormone output is diminished, and the thyroid gland passes into a resting state.

The maintenance of normal conditions in the body, so far as thyroid hormone is concerned, must therefore depend on the precise relationship between the two actions of this hormone, namely its effect on tissue metabolism on the one hand and its effect on the anterior pituitary gland on the other. The question arises as to whether it is possible to dissociate these two effects.

If such a dissociation could be brought about the results would be of considerable interest. A compound possessing the characteristic effect of thyroxine on tissue metabolism but, lacking its action on the anterior pituitary gland would, if administered exogenously, produce a greatly exaggerated stimulation of metabolism, since its own intrinsic action in this direction would be unaccompanied by the restraint of thyroid gland activity imposed by diminution of thyrotrophic hormone output. On the other hand, a compound acting on the anterior pituitary gland in the same way as thyroxine, but lacking the power of the latter to stimulate tissue

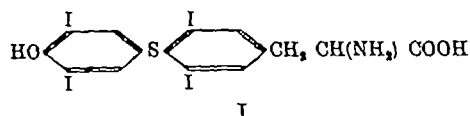
metabolism, would bring about inhibition of thyroid activity and an overall reduction in the metabolic rate.

The latter possibility is the more interesting, for, with the aid of such a compound, thyroid activity could be controlled in a more rational manner than it can be by the so called antithyroid drugs. The depression of thyroid function brought about by these drugs necessarily carries with it the likelihood of simultaneous production of physical hypertrophy of the gland, a compound having the properties discussed above should, however, diminish thyroid function, in the sense of thyroxine output, without entailing hypertrophy of the gland, its action should indeed cause a hyperactive gland, such as that existing in Graves's disease, to revert to the normal state. The therapeutic potentialities of such a compound are obvious.

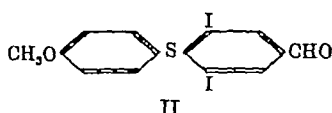
Consideration on these lines points to the desirability of examining the physiological action of compounds closely allied in chemical composition to thyroxine itself. Certain substances of this kind are already known, namely the analogues of thyroxine in which the iodine atoms are replaced by bromine or chlorine (cf Schuegraf, 1929). It was shown many years ago by Gaddum (1929-30) that 3,5,3',5' tetra-bromothyronine had the same qualitative effect as thyroxine, although in much lower degree. The action of this compound, and also that of the chlorine-containing analogue, have recently been investigated in human myxoedema (Lerman & Harington, 1948), both have proved to be capable, in appropriate doses, of restoring the metabolism of a myxoedematous patient to the normal level. In so far as these substances exhibit the characteristic peripheral effect of thyroxine, therefore, they do not seem to be promising from the present point of view, however, their action on the anterior pituitary gland remains to be investigated and the possibility exists that this may predominate over the peripheral effect, if such be the case it should be possible, by their administration at the correct level of dosage, to produce a diminution rather than an increase in the rate of metabolism.

In the meantime it has seemed desirable to make available for study quite a different type of thyroxine analogue and for this purpose the compound (I) was selected, it will be seen that this is identical with thyroxine save that sulphur replaces oxygen as

the element linking the two benzene rings It appeared likely on general grounds that the sulphur containing derivative could be synthesized on the same lines as thyroxine itself (cf Harington & Barger, 1927) So far as the early stages of the

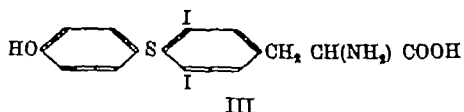


synthesis are concerned this indeed proved to be the case, and it was possible, starting with 4-methoxythiophenol and 3,4,5-triodonitrobenzene, to obtain 3,5-diiodo-4-(4'-methoxy)phenylsulphidobenzaldehyde (II) by a series of reactions precisely similar to those



employed in the thyroxine synthesis, the yields in the diphenyl sulphide series were in fact rather better than in the diphenyl ether series

Quite unexpectedly, however, the synthesis of 3,5-diiodo-4-(4'-hydroxy)phenylsulphidophenylalanine (III) from the aldehyde (II) could not be carried out



by the modified Erlenmeyer method (cf Harington & McCartney, 1927) Condensation of the aldehyde (II) with hippuric acid gave the azlactone in excellent yield, and this in turn could be smoothly converted into the corresponding benzamidocinnamic acid, but when either this compound or the azlactone itself was boiled with hydriodic acid, acetic acid and red phosphorus, elimination of the iodine atoms readily occurred and the product consisted of a mixture of partly and completely deiodinated material Many attempts were made to circumvent this difficulty by modification of the conditions, but without success, even treatment with dilute hydriodic acid in acetic acid, as employed by Lamb & Robson (1931) in the stepwise conversion of azlactones and benzamidocinnamic acids into amino acids, failed to give a satisfactory result

In the case of diiodothyronine (Harington & McCartney, 1927) the analogous conversion of the azlactone or benzamidocinnamic acid can be carried out in good yield, although even here more recent experience has shown that too prolonged boiling with the hydriodic acetic acid mixture and phosphorus causes more or less deiodination It is clear, however, that the lability of the iodine atoms in the

corresponding diphenyl sulphide derivative is much greater, and an alternative route from the aldehyde to the amino acid had to be found

Success was attained quite simply as follows The aldehyde (II) was reduced to the corresponding alcohol by means of aluminium isopropoxide, the alcohol was converted into the chloride and the latter condensed with ethyl acetamidomalonate, appropriate treatment of the resulting ester with a mixture of hydrobromic and acetic acids gave the hydroxy-diiodoamino acid (III) in satisfactory yield and final iodination in ammoniacal solution afforded the desired thyroxine analogue (I)

In acute experiments the thyroxine analogue was non-toxic to mice in doses up to 0.5 g/kg (subcutaneous) and 0.25 g/kg (intravenous) Tested on tadpoles (*Xenopus laevis*) it accelerated metamorphosis, its activity in this respect being approximately one-fifth of that of thyroxine More detailed studies of the physiological action are now in progress

EXPERIMENTAL

3,5-Diiodo-4-(4'-methoxy)phenylsulphidobenzaldehyde 3,4,5-Triodonitrobenzene (86.5 g) was dissolved in dioxan (175 ml) and 4-methoxythiophenol (26 ml) (Suter & Hanson, 1932) was added The mixture was vigorously stirred at room temperature while NaOH (17.3 ml of 10N) was run in Heat was developed and crystalline material soon began to separate Stirring was continued for 30 min after which a sample diluted with water had a neutral reaction

Water (about 2 vol.) was then added with further stirring, the precipitate, which was first oily but soon crystallized, was collected and washed with water It was recrystallized from acetic acid, from which it separated in long bright yellow needles, m.p. 140° The melting point was unchanged by further crystallization from methyl ethyl ketone Yield 78 g (92%) (Found S, 6.2, I, 49.7 C₁₃H₉O₃NSI₂ requires S, 6.2, I, 49.5%)

3,5-Diiodo-4-(4'-methoxy)phenylsulphidoaniline Stannous chloride (110 g of the dihydrate) was suspended in acetic acid (500 ml) and brought into solution by saturation with HCl while being stirred and heated to about 70° on a water bath The above nitro compound (73.5 g) was then added as a suspension in acetic acid (250 ml) The water bath was brought to the boil, vigorous stirring and rapid introduction of HCl being continued. The reaction appeared to be complete in about 45 min. as shown by saturation with HCl and no further separation of stannic chloride, after a further 15 min the mixture was cooled and the stannous chloride of the base collected and washed with cold acetic acid. It was then ground up and shaken well with NaOH solution (1500 ml of 3N) and the base collected and washed with water The base was dissolved in ether (3000-3500 ml.) and the ethereal solution washed with 200 ml of 2N NaOH, dried over NaOH pellets and saturated with HCl The precipitated hydrochloride was collected, washed with ether and dried. The yield was 63 g (84%) After recrystallization from ethanol containing a little HCl it had m.p. 166-168° (Found N, 2.5, S, 5.6 C₁₃H₁₁ONS₂·HCl requires N, 2.7, S, 6.2%)

The free base was prepared by dissolving the hydrochloride in absolute ethanol, adding excess of ammonia to the hot solution and diluting with water. After recrystallization from butanol it formed colourless fern shaped crystals, m.p. 177° (Found N, 2.7, S, 6.9 $C_{13}H_{11}ONSi_2$ requires N, 2.9, S, 6.6%)

3.5 Duodo-4(4'-methoxy)phenylsulphidobenzonitrile. A solution of cuprous cyanide was prepared by mixing NaCN (213 g) dissolved in water (490 ml.) with $CuSO_4 \cdot 5H_2O$ (250 g) in water (970 ml). The hydrochloride of the above base (52 g) was powdered and suspended in acetic acid (520 ml.), the stirred suspension was treated with amyl nitrite (15 ml.) which was added quickly, the solid passed into solution within a few minutes. The solution thus prepared was added slowly with stirring to the cooled cuprous cyanide and the mixture warmed to about 80° to complete the reaction. The precipitate was collected, washed with water and boiled out with three successive quantities of chloroform. The chloroform extracts were dried and evaporated to a low bulk, when crystallization set in. After thorough cooling the crystals were collected and recrystallized from methyl ethyl ketone, the solution being cooled in a freezing mixture. There was thus obtained 28.4 g (55%) of a product, melting at 149° after some sintering, which was pure enough for the next reaction. After further recrystallization from methyl ethyl ketone the compound formed pale yellow hexagonal prisms, m.p. 150° (Found N, 2.5, S, 6.5, I, 51.4 $C_{14}H_{10}ONSi_2$ requires N, 2.8, S, 6.4, I, 51.5%)

3.5 Duodo-4(4'-methoxy)phenylsulphidobenzaldehyde (II) $SnCl_2$ (60 g) was suspended in dry ether (220 ml) and brought into solution by stirring and saturating with HCl. A solution of the above nitrile (28.15 g) in dry chloroform (75 ml.) was added, stirring and introduction of HCl being continued for 5 hr. After standing overnight the mixture, which consisted of an upper pale yellow and a lower deep orange coloured layer, was treated with crushed ice and evaporated *in vacuo* until all the organic solvents were removed. The solid residue was heated with dilute HCl to the boiling point and, after cooling, the crude aldehyde was taken up in chloroform, the chloroform solution was shaken with aqueous $Na_2S_2O_5$ (300 ml. of 30% (w/v)) and the bisulphite compound which separated was collected, washed with ether and decomposed by warming with dilute H_2SO_4 . The aldehyde was crystallized from acetic acid from which it separated in large pale yellow needles, m.p. 134° (unchanged by further recrystallization). The yield was 18.6 g (86%) (Found S, 6.3, I, 50.6 $C_{14}H_{10}O_2Si_2$ requires S, 6.45, I, 51.2%)

Azlacone. The above aldehyde (11.1 g), hippuric acid (4 g) and fused sodium acetate (11.1 g) were ground up together, the mixture was treated with acetic anhydride (70 ml.) and heated on the boiling water bath for 15 min with intermittent stirring. After cooling, the mixture was stirred with a large volume of water containing some H_2SO_4 until the acetic anhydride was decomposed. The product was collected, washed with water and dried. The yield was 13.9 g (97%) of practically pure azlacone, m.p. 224°. After recrystallization from acetic acid, in which it is very sparingly soluble, it formed yellow needles, m.p. 228° (Found C, 43.0, H, 2.35, S, 5.0 $C_{23}H_{18}O_3NSi_2$ requires C, 43.2, H, 2.5, S, 5.0%)

3.5 Duodo-4(4'-methoxy)phenylsulphidobenzamido cinnamic acid. The azlacone (9.5 g) was added to a boiling mixture of ethanol (950 ml) and 5N NaOH (50 ml.), the solid passed

rapidly into solution and boiling was continued for 10 min after this had happened. The solution was acidified while hot with concentrated HCl and diluted with an equal volume of water. This caused separation of the product in gelatinous form. The acid was brought into solution with ammonia and the boiling solution again acidified, boiling was continued for a few minutes after which the precipitated acid was almost completely crystalline. The yield of acid, collected after cooling, was quantitative. Recrystallized from aqueous dioxan it formed bunches of pale yellow needles, m.p. 245° (decomp) (Found S, 4.7 $C_{23}H_{17}O_6NSi_2$ requires S, 4.9%)

As already indicated, all attempts to convert either this acid or the azlacone into 3.5 duodo-4(4'-methoxy)phenyl sulphidophenylalanine were frustrated by the ease with which the iodine atoms were eliminated on treatment with hydriodic acid and red phosphorus.

3.5 Duodo-4(4'-methoxy)phenylsulphidobenzyl alcohol. 3.5 Duodo-4(4'-methoxy)phenylsulphidobenzaldehyde (5 g) was heated to gentle boiling under a reflux air condenser with a mixture of aluminum isopropoxide (20 ml. of approximately N solution in isopropanol), toluene (10 ml) and isopropanol (10 ml.), a slow stream of N_2 was bubbled through the solution until evolution of acetone ceased (about 3½ hr). The solution was evaporated to dryness *in vacuo* and the residue was ground up with dilute HCl, collected and washed with water. The moist filter cake was crystallized from acetic acid, in which it was very soluble, the product, which separated in colourless prismatic needles, m.p. 132°, amounted to 4.5 g (90%) (Found S, 6.5, I, 50.3 $C_{14}H_{12}OSi_2$ requires S, 6.4, I, 51.0%)

3.5 Duodo-4(4'-methoxy)phenylsulphidobenzyl chloride. The above alcohol (3 g) was dissolved in warm dry chloroform (15 ml.). The solution was cooled in ice water and to the resulting crystalline paste PCl_5 (1.5 g) was added. The mixture was shaken with continued cooling until practically all the solid had passed into solution, it was then kept at room temperature for 2 hr. A small amount of precipitate was removed by filtration and the filtrate was diluted with about 2 vol. of light petroleum (b.p. 60–80°), thus produced a slight brown flocculum which was removed. The filtrate was then washed with water, $NaHCO_3$ solution (thoroughly) and again with water, it was dried over $CaCl_2$ and evaporated *in vacuo*. The residue crystallized almost at once, it was recrystallized from a small volume of acetic acid from which it separated in colourless rhombs, m.p. 110°. One further recrystallization raised the m.p. to 112°. The yield was 2.5 g (83%) (Found S, 6.3, I, 48.7 $C_{14}H_{11}OSCl_2$ requires S, 6.2, I, 49.1%)

Ethyl 3.5 duodo-4(4'-methoxy)phenylsulphidobenzylacetamidomalonate. A solution of sodium (0.1 g) in ethanol (5 ml.) was prepared and, after cooling, ethyl acetamidomalonate (0.84 g) was introduced, there was then added a solution of the above chloride (2 g) in dioxan (5 ml). The mixture was boiled under reflux, separation of NaCl soon began, and the reaction was complete in 2½ hr. On cooling and dilution with water an oily precipitate was produced which soon crystallized, this was collected, washed with water and recrystallized from ethanol. The ester formed colourless prismatic needles, m.p. 138–140°. The yield was 2.25 g (83.5%) One further recrystallization from ethanol raised the m.p. to 144° (Found C, 39.8, H, 3.8, S, 4.5, I, 36.2 $C_{23}H_{22}O_6NSi_2$ requires C, 39.6, H, 3.6, S, 4.6, I, 36.4%)

3 4 *Diiodo-4(4' hydroxy)phenylsulphidophenylalanine* (III) Prolonged boiling of the above ester with mixtures of hydrobromic and acetic acids caused considerable darkening with formation of tar and correspondingly poor yields of the desired amino acid. It was assumed that of the three hydrolytic reactions occurring, deacetylation, de-esterification and demethylation, the last would be the slowest, an experiment was therefore carried out to determine the minimum time required for this reaction. In this experiment anisic acid was boiled under reflux with forty parts of a mixture of equal volumes of HBr (6N) and acetic acid, samples being withdrawn at intervals for estimation of the phenol (*p* hydroxybenzoic acid) content. The results showed that demethylation was substantially complete (95-100%) in 2 hr.

The ester (6 g) was therefore boiled under reflux for 2 hr with a mixture of HBr (120 ml of 6N) and acetic acid (120 ml). The solution was evaporated to dryness *in vacuo* and the largely crystalline residue was boiled with water (about 250 ml) and filtered hot through a wet paper to remove tar. The filtrate was brought to the boil and cautiously neutralized to about pH 5.0 with sodium acetate. The heavy granular precipitate which separated was collected after cooling, washed with water and redissolved in a mixture of 80 ml ethanol and 20 ml N NaOH. On boiling this solution and adding acetic acid rapid separation of the amino acid occurred in tufts of colourless needles, m.p. 262° (decomp). The yield was 3.05 g (85%). (Found C, 33.0, H, 2.4, S, 6.1, I, 46.9. $C_{15}H_{13}O_5NSI_2$ requires C, 33.25, H, 2.4, S, 5.9, I, 47.0%). The compound gave a ninhydrin reaction with a reddish hue, and a positive Millon reaction. It was practically insoluble in water and only very sparingly soluble in dilute aqueous mineral acids.

3 5 *Diiodo-4(3' 5' diiodo-4' hydroxy)phenylsulphidophenylalanine* (I) The diiodoamino acid (3.6 g) was dissolved in ammonia (144 ml. of sp. gr. 0.880), this solution was treated drop by drop with I_2 in KI (6.2 ml. of 4.31N). A small amount of tarry material separated towards the end of the reaction. After all the iodine had been added the solution was evaporated to dryness *in vacuo* and the residue, which was already partly crystalline, was dissolved in boiling 0.1N Na_2CO_3 (about 1000 ml.), a little insoluble matter was removed by filtration and the filtrate was brought to about pH 5.0 at the boiling point by addition of dilute HCl. The partly crystalline precipitate was collected after cooling and dissolved in a mixture of 100 ml ethanol and 40 ml. N NaOH, the solution was

brought to the boil and cautiously treated with acetic acid. Just before neutrality was reached a pigmented flocculent precipitate separated, this was removed by filtration as rapidly as possible, the filtrate was brought to the boil and the acidification with acetic acid completed. The amino acid separated in rosettes of tiny needles. The yield was 3.6 g (68%). For complete purification the crystallization was repeated as above, again with removal of a slight pigmented flocculum, and the product was then obtained in sheaves of small but well formed needles which were almost colourless, m.p. 228° (decomp). (Found C, 23.5, H, 1.6, S, 4.0, I, 64.0. $C_{15}H_{11}O_5NSI_4$ requires C, 22.7, H, 1.4, S, 4.0, I, 64.0%). The compound resembled thyroxine closely in its general physical properties and solubilities, it gave with intensity the colour reaction with nitrous acid and ammonia which is given by thyroxine and many other *o*-diiodophenols.

SUMMARY

1 The possibility is discussed of controlling thyroid function by means of compounds inhibiting the output of thyrotrophic hormone from the anterior pituitary gland.

2 It is pointed out that such compounds may occur among chemical analogues of thyroxine. The synthesis is described of 3 5-diiodo-4(4' hydroxy)-phenylsulphidophenylalanine, a sulphur containing analogue of thyroxine, which is designed for the test of this hypothesis.

After this synthesis had been planned and begun I learnt by chance that Dr Paul Block, junr., had done some preliminary experiments on similar lines which circumstances had compelled him to interrupt. Since Dr Block did not have the particular theoretical interest in the work which is indicated by the introduction to this paper, and since the early resumption of his experiments was impossible, it was agreed that the synthesis should be completed in my laboratory. I wish to express my appreciation of Dr Block's attitude in this matter, and also of his generosity in giving me valuable information about the most favourable conditions for carrying out the first reaction.

I am indebted to Dr W. L. M. Perry for carrying out the toxicity tests and to Dr A. S. Parkes for the tests on tadpoles. My thanks are also due to Mr W. Hickford for technical assistance.

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The Action of Alkalis on Wool

3 THE ACTION OF ALKALIS ON DEAMINATED WOOL

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Following the isolation of lanthionine from alkali-treated wool by Horn, Jones & Ringel (1941), Cuthbertson & Phillips (1945) demonstrated that two major reactions of the combined cystine occurred during treatment of wool with dilute alkalis, one leading to the formation of lanthionine and the other by a reaction analogous to one previously studied by Bergmann & Stather (1926) leading to the production of combined α aminoacrylic acid. They also found that reactions previously suggested by Speakman (1936) and Phillips (1936), giving rise to $-S-NH-$ and $-CH=N-$ linkages in the fibre, did not occur.

These latter linkages had the great merit, however, of explaining certain aspects of the physical behaviour of wool fibres which could not be readily explained on the basis of the two reactions formulated by Cuthbertson & Phillips (1945). Thus, if a wool fibre is extended in cold water and boiled in a mildly alkaline buffer for some time, it remains permanently elongated and will not return to its original length even when it is boiled in water in the absence of tension. If a deaminated fibre is similarly treated, however, far from acquiring a permanent set, it will 'supercontract' in boiling water to a length less than its original unstretched length. Speakman's chemical theory of set explained the setting of the untreated fibre as being due to the formation of stabilizing $-S-NH-$ linkages, whilst the impossibility of forming such linkages in deaminated fibres leads to supercontraction. If the setting of wool fibres by treatment with dilute alkali is attributed to the formation of lanthionine cross linkages, no explanation is afforded of the failure to set and the supercontraction of deaminated fibres. Two hypotheses may, however, be considered: (a) the action of alkali on deaminated fibres is different in untreated and deaminated wool, or (b) the mechanism of set is fundamentally physical rather than chemical. Evidence is presented in the present paper to show that the combined cystine of deaminated wool does behave in a different manner towards alkalis. Under the conditions used by Speakman in his investigation of the physical properties of fibres, however, the difference in reactivity is slight, and to this extent the results support the physical mechanism of set proposed by Blackburn & Lindley (1948).

METHODS

The wool used was a commercially scoured and dry combed merino wool of 64's quality purified by successive extraction with benzene, ethanol and water. All analyses were carried out on wool conditioned in a controlled atmosphere at 21° and 65% relative humidity, but are recorded as percentages of the dry weight. Total S was determined by a modification of the Benedict-Denis method (Barritt, 1934), SO_4^{2-} S and S oxidizable to SO_4^{2-} were determined on the acid hydrolysate of the wool (1 g hydrolyzed for 4 hr with 5N HCl) by Blumenthal & Clarke's (1935) method, thiol and disulphide S by the method of Shinozuka (1935a, b), using a Spekker absorptiometer, methionine S was calculated from a determination of 'volatile iodide' using Baernsten's (1936) technique. Total N was determined by micro Kjeldahl procedure, amino N on intact wool by the method of Lindley & Phillips (1947), arginine by Vickery's (1940) method. Cysteic acid was semi quantitatively estimated by comparing the intensity of colour and spot size given on a filter paper strip by a known amount of wool hydrolysate (250 μ g) with known amounts of cysteic acid using phenol 0.1% NH_3 coal gas (Consden, Gordon & Martin, 1944, 1946). For lanthionine, a similar method was used after removal of aspartic and glutamic acids by Amberlite IR 4 at pH 3-4 (Consden, Gordon & Martin, 1948) using 5 μ l of hydrolysate equivalent to 80 μ g of wool. Some interference from cystine still occurs after removal of glutamic and aspartic acid, however, and in some of the later determinations the hydrolysate was treated with Br_2 . This oxidizes cystine to cysteic acid and thus prevents interference. The sulphoxide of lanthionine also formed by the action of Br_2 has a somewhat lower R_F value than lanthionine so that the danger of overlapping with the serine spot no longer exists. (It is hoped to develop a quantitative method for lanthionine on these lines using starch columns (Elsden & Syngé, 1944).) The wool was deaminated by the method of Speakman & Stott (1934).

Alkali treatment of the deaminated wools. Deaminated wool (3 g) was boiled under reflux for varying periods in 375 ml of the following buffers: pH 8.2 (8.06 g boric acid plus 6.7 g sodium borate/l, observed pH 8.22), pH 9.18 (0.05M sodium borate, observed pH 9.18), pH 10.0 (2.58 g boric acid and 4.4 ml of 40% (w/v) NaOH in 1 l, observed pH 10.4, solution was brought to pH 10 by addition of HCl). In each case the wool was washed with water, dried and conditioned before analysis.

The measurement of the setting properties of deaminated wool was made in a frame similar to that used by Speakman (1936).

RESULTS

Analysis of deaminated wool

Table 1 lists analyses of untreated wool and wool which had been deaminated up to a maximum of four times under the conditions used by Speakman & Stott (1934). The results show that some oxidation of cystine S has occurred in the first deamination, but

filter paper confirmed the fact that lysine was absent from a hydrolysate of once deaminated wool, but a strong spot corresponding to α amino- ϵ -hydroxycaproic acid was observed. Lindley & Phillips (1947) had no difficulty in acetylating all the amino groups of wool at room temperature. The arginine content of once deaminated wool was found to be 4.8% compared with 10.2% for untreated wool. It would

Table 1 *The S distribution and amino N content of wool subjected to repeated deamination treatments*

No of deamination treatments	Total S (%)	Thiol + disulphide S (%)	Br-oxidizable S + SO ₄ ²⁻ as S (%)	Methionine S (%)	Amino N (mg/g wool)
0	3.68	3.05	0.26	0.15	3.05
1	3.67	2.51	0.22	0.15	1.27
2	3.67	2.49	0.36	0.15	1.26
3	3.63	2.49	0.34	0.15	1.26
4	3.49	2.43	0.33	0.15	1.09

that little further attack takes place on repeated deamination. It can also be seen that the amino N of the wool is reduced substantially, but not completely, by repeated deamination. This is somewhat surprising since the method of calculation (extrapolation back of the straight line portion of the

appear, therefore, either that the method of calculating the amino N figures does not apply to deaminated wool or that the peptide bonds of deaminated wool are photosensitive. It was noted that tyrosine was absent from two dimensional chromatograms of deaminated wool.

It will be seen that once deaminated wool contains 0.79% S unaccounted for either as cystine, methionine, sulphate S or Br oxidizable S ('non disulphide S'), compared with 0.22% non-disulphide S for untreated wool. Of this S, 0.2% was found to be present as cysteic acid formed by oxidation of cystine during deamination, and both untreated and deaminated wools contained about 0.2% of lanthionine S, but the remaining 0.4% of non-disulphide S of deaminated wool remains unidentified.

Physical properties of deaminated fibres

Speakman (1936) found that deaminated fibres spontaneously contracted to a length less than their original length on immersing in boiling 0.05M borax solution. In order to confirm that our deaminated fibres behaved similarly, two deaminated and two untreated fibres were measured before and after boiling in 0.05M-borax solution for 10 min. The deaminated fibres showed supercontractions of 30 and 23%, compared with 1.6 and 1.9% for the untreated fibres, thus confirming Speakman's observations.

Speakman also noted that deaminated fibres would not take a permanent set in steam or borax, thus, two fibres, one untreated and the other deaminated, were extended 36% and steamed in the stretched condition for 6 hr. Steaming was then interrupted and both fibres showed no recovery. On steaming in the absence of tension, however, the deaminated fibre rapidly contracted, eventually (480 min) being 45% shorter than the original length, whereas the untreated fibre still remained

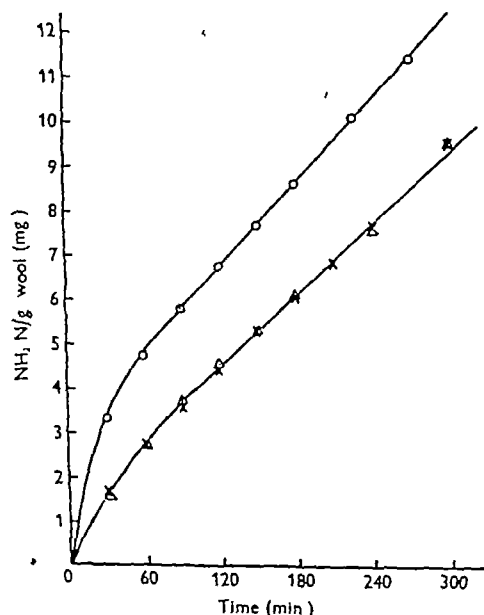


Fig. 1 The rate of liberation of nitrogen from deaminated wool on treatment with nitrous acid. \odot — \odot , untreated wool, \times — \times , deaminated wool, \triangle — \triangle , thrice deaminated wool.

curve relating time and nitrogen evolved (Rutherford, Harris & Smith, 1937)) suggests that liberation of the amino N (excluding arginine) is complete in 30 min treatment with nitrous acid (cf Fig. 1). Moreover, two dimensional chromatography on

permanently elongated by 20%. Figs 2 and 3 show the setting properties of our untreated and deaminated fibres (a) on setting in boiling water for 3 hr and (b) in 0.05M-borax for 30 min at 100°.

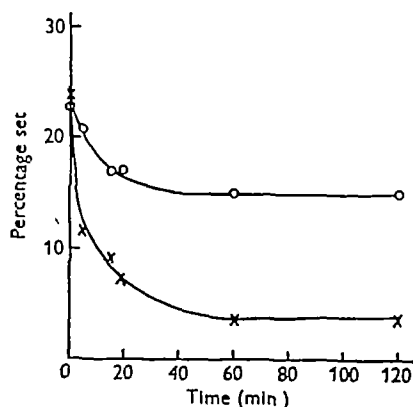


Fig 2 The rate of release of set of untreated and deaminated wool fibres, in boiling water, after being set-extended in water at 100° for 3 hr. \bigcirc — \bigcirc , untreated wool, \times — \times , deaminated wool

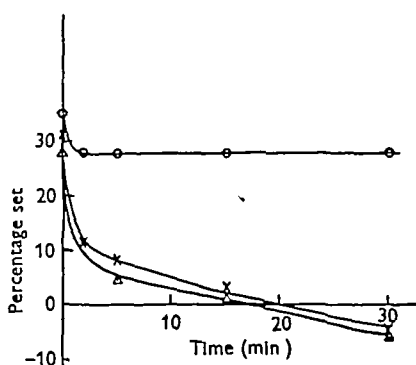


Fig 3 The rate of release of set of untreated and deaminated wool fibres in boiling water after being set-extended in boiling 0.5M borax for 30 min. \bigcirc — \bigcirc , untreated wool, \times — \times , deaminated wool, Δ — Δ , thrice deaminated wool

respectively. It will be seen that our deaminated fibres retain more set than Speakman's (Cotswold) fibres. That this was not due to incomplete deamination was shown by the similarity in properties of once and thrice deaminated wool. It thus probably represents a difference between the wools used by Speakman and that used in the present investigation. Other differences of this kind have already been observed (Blackburn & Lindley, 1948).

The S distribution of deaminated wools after boiling with buffers of pH 8.2–10

The S distribution of deaminated wool which has been treated for different periods of time with boiling buffer solutions of pH 8.2, 9.2 and 10.0 is shown in

Table 2. From these analytical results the loss of disulphide S, and sulphur loss are calculated as follows:

$$\text{Loss of disulphide S} = [\text{thiol S} + \text{disulphide S in deaminated wool}] - [\text{thiol S} + \text{disulphide S in alkali treated deaminated wool}],$$

and

$$\text{S loss} = [\text{total S} - \text{Br oxidizable S} + \text{sulphate S in deaminated wool}] - [\text{total S} - \text{Br oxidizable S} + \text{sulphate S in alkali treated deaminated wool}].$$

These values are shown in Table 3, together with the results of semi-quantitative lanthionine estimations carried out on some of the treated wools. The cysteic acid content, equivalent to about 0.2% S, remained unchanged after alkali treatment.

Compared with untreated wool (Cuthbertson & Phillips, 1945; Lindley & Phillips, 1945) the results are complicated in this case by the occurrence of substantial quantities of unidentified S in the fibre. The simplest explanation of the results, however, would seem to be that the cystine breaks down in the normal manner to give both lanthionine and combined α -aminoacrylic acid, whilst the unidentified S partly remains unchanged, and partly decomposes with complete loss of sulphur from the fibre. Thus the maximum lanthionine produced in deaminated wool (c. 0.8% S) plus half the unidentified S (0.6–2.1 or 0.3%) is equivalent to the 1.0–1.2% lanthionine S which is the maximum normally found for virgin wool after alkali treatment.

Table 4 gives the amino N content of some alkali-treated, deaminated wools. Subject to the reservations noted above, the results are interesting in showing an increase in amino N on alkali treatment in contrast to the decrease found in alkali-treated virgin wool by Cuthbertson & Phillips (1945). It would seem possible, therefore, that the peptide bonds in deaminated wool are less stable to alkali than is the case with untreated wool. Although the values are small they may, nevertheless, imply considerable changes in the average length of peptide chains and could thus greatly influence the physical properties of deaminated fibres.

The S distribution of deaminated wool before and after treatment with dilute alkalis at room temperature

A new batch of deaminated wool was used for these experiments. Its S distribution, before and after treatment with solutions of sodium hydroxide and barium hydroxide, is shown in Table 5, whilst Table 6 lists the derived S distributions of these wools.

These results contrast very sharply with those of Cuthbertson & Phillips (1945) on virgin wool, in

Table 2 *The S distribution of deaminated wool before and after treatment with boiling buffer solutions of pH 8.2-10*

Nature of treatment		Thiol + disulphide S (%)	Br-oxidizable S + SO ₄ as S (%)	Methionine S (%)	Total S (%)
pH of buffer	Time of treatment (hr)				
—	0	2.51	0.22	0.15	3.87
8.2	0.5	2.25	0.23	0.15	3.37
	1	2.06	0.29	0.15	3.34
	2	1.80	0.27	0.15	3.29
	3	1.51	0.27	0.15	3.11
	4	1.19	0.32	0.15	3.02
9.18	5	1.08	0.34	0.15	2.89
	0.25	1.68	0.70	0.15	3.28
	0.5	1.19	0.73	0.15	3.08
	1	0.72	0.65	0.15	2.69
	2	0.54	0.64	0.15	2.46
10.0	4	0.54	0.33	0.15	2.17
	0.25	1.52	0.60	0.15	3.07
	0.5	0.59	0.45	0.15	2.70
	1	0.45	0.45	0.15	2.53
	2	0.42	0.39	0.15	2.18
Before deamination		3.05	0.26	0.15	3.68

Table 3 *The non-disulphide S, disulphide S loss, S loss, and lanthionine content of deaminated wool after boiling with buffers of pH 8.2-10*

Nature of treatment		Non disulphide S (%)	Increase in non disulphide S on alkali treatment (%)	Disulphide S loss (%)	S loss (%)	Lanthionine S (%)
pH of buffer	Time of treatment (hr)					
—	0	0.79	0	0	0	0.20
8.22	0.5	0.74	-0.05	0.26	0.31	0.35
	1	0.84	0.05	0.45	0.40	—
	2	1.07	0.17	0.71	0.43	—
	3	1.18	0.39	1.00	0.61	—
	4	1.37	0.58	1.32	0.75	—
9.18	5	1.32	0.53	1.43	0.90	0.80
	0.25	0.75	-0.04	0.83	0.87	0.45
	0.5	1.01	0.22	1.32	1.10	—
	1	1.17	0.38	1.79	1.48	—
	2	1.14	0.35	1.97	1.63	0.70
10.0	4	1.15	0.36	1.97	1.61	—
	0.25	0.80	0.01	0.99	0.98	0.40
	0.5	1.51	0.72	1.92	1.20	—
	1	1.48	0.69	2.06	1.32	—
	2	1.22	0.43	2.09	1.66	—
Before deamination		0.22	—	—	—	0.20

which lanthionine was rapidly formed. A slow breakdown, presumably of the Bergmann & Stather (1926) type, occurred in the baryta treatment, but was not evident at all in treatments with sodium hydroxide until the normality reached 1.0. The present results in deaminated wool, however, show that the major reaction occurring throughout is of the Bergmann & Stather type with complete loss of sulphur from the fibre, and the results with baryta suggest that this occurs not only with the cystine but also with the unidentified S.

Table 4 *The amino N content of deaminated wool before and after boiling with buffers of pH 8.2-10*

Nature of treatment		Amino N content (mg/g wool)
pH of buffer	Time of treatment (hr)	
—	0	1.27
8.22	3	2.30
9.18	2	2.30
10.0	0.5	2.13
Before deamination		3.05

Table 5 *The S distribution of deaminated wool before and after treatment with dilute solutions of NaOH and Ba(OH)₂ at room temperature*

Nature of treatment		Time of treatment (hr)	Thiol + disulphide S (%)	Br oxidizable S + SO ₄ as S (%)	Methionine S (%)	Total S (%)
Reagent	—					
—	—	0	2.52	0.47	0.15	3.67
0.1N NaOH	—	0.5	2.27	0.35	0.15	3.33
	—	2.5	1.82	0.46	0.15	3.15
	—	4.5	1.58	0.52	0.15	2.87
	—	7	1.23	0.46	0.15	2.68
	—	16.5	0.88	0.60	0.15	2.35
	—	24	0.82	0.51	0.15	2.24
	—	48	0.73	0.51	0.15	2.15
0.1N NaOH	—	1	2.11	0.63	0.15	3.15
0.5N NaOH	—	1	0.81	0.40	0.15	2.19
1.0N NaOH	—	1	0.44	0.32	0.15	1.68
0.385N Ba(OH) ₂	—	0.25	1.47	0.32	0.15	2.40
	—	0.5	1.28	0.53	0.15	2.26
	—	1	0.82	0.58	0.15	2.25
	—	2	0.61	0.58	0.15	1.68
	—	4	0.43	0.34	0.15	1.08
	—	6	0.24	0.34	0.15	1.01

Table 6 *The non-disulphide S, disulphide S loss, S loss and lanthionine content of deaminated wool after treatment with dilute NaOH and Ba(OH)₂ solutions at room temperature*

Nature of treatment		Time of treatment (hr)	Non disulphide S (%)	Increase in non disulphide S on alkali treatment (%)	Disulphide S loss (%)	S loss (%)	Lanthionine S (%)
Reagent	—						
—	—	0	0.53	0	0	0	0.20
0.1N NaOH	—	0.5	0.56	0.03	0.25	0.22	0.30
	—	2.5	0.72	0.19	0.70	0.51	—
	—	4.5	0.62	0.09	0.94	0.85	—
	—	7	0.84	0.31	1.29	0.98	0.40
	—	16.5	0.72	0.19	1.64	1.45	—
	—	24	0.76	0.23	1.70	1.47	—
	—	48	0.76	0.23	1.79	1.56	—
0.1N NaOH	—	1	0.26	-0.27	0.41	0.68	—
0.5N NaOH	—	1	0.83	0.30	1.71	1.41	—
1.0N NaOH	—	1	0.77	0.24	2.08	1.84	0.70
0.385N Ba(OH) ₂	—	0.25	0.46	-0.07	1.05	1.12	—
	—	0.5	0.30	-0.23	1.24	1.47	—
	—	1	0.70	0.17	1.70	1.53	—
	—	2	0.34	-0.19	1.91	2.10	—
	—	4	0.16	-0.37	2.09	2.46	—
	—	6	0.28	-0.25	2.27	2.53	—

DISCUSSION

The results obtained in the present investigation are important in providing another example of how the reactivity of a particular group (i.e. the disulphide bond of cystine) can be altered by modifications of other groups in the protein molecule (cf. Lindley & Phillips, 1947). The observation that lysine is absent from hydrolysates of deaminated wool, but that α -amino ϵ -hydroxycaproic acid is present is of some importance in confirming accepted views on the mode of linkage of lysine. The spot corresponding to

α -amino- ϵ -hydroxycaproic acid was only found in hydrolysates of deaminated wool and was not noted in alkali treated, deaminated wool.

Nothing definite is known of the fate of arginine during deamination of wool. The first product might be citrulline which, according to Wada (1933), would form proline in boiling hydrochloric acid. Pure citrulline, however, under our conditions of hydrolysis (5N-HCl, 24 hr.), was found by paper chromatography to be partially changed to ornithine, without formation of proline. No evidence of ornithine was found in the chromatograms of hydrolysates of

deaminated wool Another possible product of deamination of arginine, α amino δ hydroxyvaleric acid, is also stated by Sorensen (1905) to form proline on acid hydrolysis (conc hydrochloric acid at 150°) Proline estimations were, therefore, carried out on hydrolysates of deaminated and untreated wool, using partition chromatography of acetamido acids on silica gel (Gordon, Martin & Syngo, 1943, Tris tram, 1946) No increase was found in the proline content of the hydrolysate of the deaminated wool Thus the mechanism of breakdown of the combined arginine of the wool under these conditions remains obscure

The results present a number of interesting points specifically concerned with the structure of wool So far as is known at present, reagents which cause supercontraction of unstretched virgin wool (i.e. α keratin) do so by fission of the disulphide bonds of cystine when cleavage is coupled with thermal agitation The sole exception to this is potassium cyanide solution which Cuthbertson & Phillips (1945) found to produce lanthionine, but which Speakman (1936) claimed caused supercontraction of human hair Numerous experiments in this laboratory, however, have failed to confirm Speakman's observation, and we have never obtained supercontraction of wool fibres or hair in cyanide solution Deaminated wool would, however, seem to provide a genuine exception to this rule since it supercontracts readily in borax solution at 100° with formation of substantial quantities of lanthionine Our deaminated fibres, for instance, supercontracted by 20–30% in 10 min in boiling borax solution, reference to Tables 2 and 3 shows that after 15 min at this pH and temperature the deaminated wool still contains 1.7% cystine S and 0.45% lanthionine S Compared with deaminated wool, this represents a decrease of 0.55% S and, even compared to untreated wool, a loss of only 1.1% S present as covalent cross linkages The work of Carter, Middlebrook & Phillips (1946) makes it improbable that such a degree of sulphur breakdown would lead to supercontraction

The results on the setting of deaminated fibres are also difficult to reconcile with the chemical theory of setting Thus, comparison of the present results with those on the action of alkalis on untreated wool (Lindley & Phillips, 1945) shows that the course of the reaction is not greatly influenced by the prior deamination treatment so far as boiling in weakly alkaline buffers are concerned, except to the extent that cystine S is destroyed by the deamination This seems to be fairly constant and amounts to not more than 0.55% of cystine S which could form 0.27% of lanthionine S, or about 25% of the maximum amount of lanthionine produced in wool in mildly alkaline buffers at 100° Whilst it is possible that this 25% may exert a decisive influence on the

physical properties of the wool fibre, it seems probable in view of evidence from other sources (Blackburn & Lindley, 1948) that cystine reactivity does not play the decisive role in the mechanism of the setting of wool fibres in mildly alkaline buffers which has been attributed to it by Speakman (1936)

With the abandonment of the $-\text{S}-\text{NH}-$ and lanthionine linkage as the important factors in the setting of wool in mildly alkaline buffers at 100° , the confirmation in the present work of Speakman's findings that deamination profoundly affects the setting properties raises another very important point with regard to wool structure Thus Speakman & Hirst (1933) suggested that the basic and acid side chains of wool were present as salt linkages between peptide chains, and the stability of the wool molecule has been regarded by Speakman as being primarily due to these salt linkages and covalent sulphur bonds On this view, therefore, and accepting the suggestion that sulphur reactivity does not play a decisive part in setting under these conditions, it might be possible to regard set as being due to breakdown of the original salt linkages followed by reformation of new ones in the extended state Such a hypothesis, however, implies that masking of the acidic side chains would affect the setting properties of wool in the same way as modification of the basic side chains by deamination In point of fact, Blackburn & Lindley (1948) found that wool in which the free carboxyl groups were esterified by treatment with methylating agents behaved in exactly the opposite manner to deaminated wool, i.e. their setting properties were enhanced, and they showed a diminished tendency to supercontract in bisulphite solution Apart from the possibility that the results on deaminated wool are due to some effect of nitrous acid on groups other than the basic side chains, two further possibilities may be borne in mind (1) the amino groups play a decisive part in set by forming a stable chemical cross link of unknown character, or (2) the basic and acidic side chains of wool have entirely separate and distinct functions in the structure of wool, i.e. the salt-linkage hypothesis must be abandoned The first hypothesis is rendered very unlikely by the observation of Cuthbertson & Phillips (1945) that the reaction of wool with nitrous acid is little affected by prior alkali treatment The second suggestion is supported to some extent by the regularities in basic amino acid composition found in keratins by Block (1939), whilst present (admittedly meagre) evidence suggests no such regularity of the dicarboxylic acid contents of keratins With the apparent demolition of the long parallel peptide chain theory of wool by Farrant, Rees & Mercer (1947), the field would now seem to be clear for an entirely new synthesis of our knowledge of the structure of wool

SUMMARY

1 The reaction of the sulphur of deaminated wool with alkalis has been studied

2 It has been found that, excluding the cystine destroyed during deamination, the deaminated wool behaves very similarly to untreated wool with mildly alkaline buffers at 100°, in particular lanthionine is formed

3 With stronger alkalis at room temperature, however, deaminated wool behaves in an entirely

different manner from untreated wool and no lanthionine is formed

4 An examination of some of the physical properties of deaminated wool has been made which has in general confirmed earlier observations of Speakman (1936)

5 Difficulties in reconciling the physical properties and the present chemical evidence with current views of wool structure are discussed

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The Effects of Progressive Nutritional Hypoproteinaemia on the Extracellular-Fluid Phase and Plasma Colloid Osmotic Pressure in Rats

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Many theories have been advanced to explain the mechanism of oedema formation under conditions of malnutrition. It is widely accepted that the main factor concerned is a deficiency of dietary protein, which in turn results in the depletion of the proteins of the body and particularly of those of the blood plasma. According to Starling's (1895) hypothesis a decrease in the plasma proteins results in a fall of the plasma colloid osmotic pressure, a decreased

ability to retain water in the capillaries, and a diffusion of fluid into the tissues. Support for the accepted theory has been provided both by animals fed on a protein-deficient diet (Kohman, 1920, Frisch, Mendel & Peters, 1929, Dicker, Heller & Hewer, 1946) and by human beings suffering from the so called 'hunger oedema' (Gounelle, Bachet & Marche, 1942, Govaerts & Legume, 1942, Mollison, 1946). However, Metcalf, Favour & Stare (1945)

claimed that feeding rats with a protein deficient diet for several days produced no more than a slight decrease of the concentration of plasma proteins, whereas prolonged feeding with a somewhat less protein-deficient diet did not affect the plasma proteins. Furthermore, among clinical cases of 'hunger oedema', reported during and after the last war, a large proportion (seventeen in forty three in the Govaerts & Lequime (1942) series) had a normal plasma protein level, with a correspondingly high colloid osmotic pressure. Finally, electrophoretic analysis of the plasma proteins of subjects suffering from experimental undernutrition did not show any hypoproteinaemia or a change in the albumin/globulin ratio (Keys, Taylor, Michelson & Henschel, 1946), even though some of them had developed visible oedema. However, an increase of the thio cyanate space (and therefore very likely of the extracellular fluid phase) was found in all these subjects whether they had developed oedema or not.

In view of these contradictory findings, the water and electrolyte distribution in skeletal muscle, in liver and in brain were studied in series of rats fed on a low-protein diet. The extracellular- and intracellular water phases of these tissues were estimated

METHODS

Experimental animals Adult male albino rats weighing 270–320 g were used.

Standard diet (ST) Control rats were given the following diet: casein 18, wheaten starch 29.5, hardened ground nut oil 9, dried yeast 11, salt mixture (Hawk & Bergeim, 1942) 3, cod liver oil 2, water 27.5%. Each animal received daily c. 20 g of this food, representing c. 60 kg cal.

Vegetable low-protein diet (turnip diet = TT) The diet was modelled on that of Kohman (1920) and of Frisch *et al.* (1929) with fresh turnips taking the place of fresh carrots, and was the same as that used by Dicker *et al.* (1946). It had the following composition: fresh turnips 85.5, wheaten starch 7, hardened ground nut oil 1.2, salt mixture 4.2, cod liver oil 2.1%. In addition, each animal received a mixture containing 30 µg aneurin, 50 µg riboflavin and 1 mg nicotinic acid. Fresh turnips contain c. 0.8% of protein.

The supply of this diet was not restricted. It was found that each animal ate c. 50 g daily, which represented an intake of about 35–40 kg cal. (The Medical Research Council War Memorandum (1945) was used for the calculation of calorific values.)

Low casein, high carbohydrate diet (LCT) The composition of this diet was as follows: casein 0.5, wheaten starch 54.0, hardened ground nut oil 7.5%, salt mixture, cod liver oil, vitamin B and tocopheryl acetate in the same amount as in the TT diet and water 31.7%.

Of this food 100 g represented 303.5 kg cal. This diet differed essentially from the TT diet in that the deficiency of calories due to the low protein content was compensated by an increased quantity of starch and fat. Each rat received about 20 g of this mixture representing a daily intake of 60 kg cal, comparable with that provided by the ST diet.

All rats had free access to water.

Procedure All rats were fed on the standard diet for about 4 weeks, before being put on an experimental diet. Series of rats on TT diet were killed after 7, 14 or 42 days. The rats on LCT diet were killed after 70 days. Immediately after death, blood samples were taken and the stomach and the small intestine (up to the caecum) were removed and weighed. The two rectus abdominis muscles and two samples of the liver were excised and the brain removed and cut sagittally. Muscle, liver and brain samples were weighed in weighing bottles and analyzed for their Cl⁻, Na, K and water content.

Analytical data collected The following were determined for each rat: (a) the content of water, Cl⁻, Na, K and N of heparymized plasma, (b) the content of water, Cl⁻, Na and K of muscle samples, (c) the content of water, Cl⁻, Na and K of liver samples, (d) the content of water, Cl⁻, Na and K of brain samples.

Preparation of muscle, liver and brain tissues for Na and K determination A known quantity of minced muscle, liver or brain was washed into a digestion tube with 10 ml of distilled water and allowed to stand overnight. The mixture was then boiled for 20 min. The boiling was repeated twice at intervals of 3 hr. After the last boiling the mixture was allowed to settle, and the supernatant liquid was decanted into a 10 ml volumetric flask. No filter was used and the device proposed by McCance & Shipp (1933) was adopted. The flask was cooled and its contents made up to the mark. It has been shown by Callow (1929) and McCance & Shipp (1933) that Na and K are quantitatively extracted from muscle tissue by this method. The same method was applied to the extraction of Na and of K from liver and brain samples.

Water determination in plasma and tissues The water content of plasma, muscle, liver and brain was obtained by drying plasma and tissue samples at 104° for 48 hr.

Estimation of plasma colloid osmotic pressure Keys & Taylor's (1935a) method and apparatus were used. Duplicate readings were made and corrected to 37°. The suspending and outer buffer fluid was made up as follows: KH₂PO₄ 0.036 mol/l, KOH 0.0395 mol/l. The pH was 7.2.

Defatting of tissues To express the water content of muscles as ml/100 g of fat-free tissue, extraction of neutral fat was carried out according to Hastings & Eichelberger (1937). The same technique of fat extraction was applied to brain samples (Manery & Hastings, 1939).

Cl⁻ determination Cl⁻ in plasma was determined by Whitehorn's (1921) method, using a 0.02N NH₄CNS solution. For muscle, liver and brain tissue, the titration was carried out after acid digestion (Hastings & Eichelberger, 1937).

Na determination Na in plasma and in tissue extracts was determined colorimetrically by the method of McCance & Shipp (1931).

K determination K in plasma and in tissues was determined by Kramer & Tisdall's (1921) method as modified by McCance & Shipp (1933), using the empirical factor 0.12.

Protein determination in plasma The plasma N content was estimated by a micro Kjeldahl method. The digestion was carried out with SeO₂, and continued for 5 hr after the clearing of the solution (Hoch & Marrack, 1945). The N was estimated colorimetrically, after nesslerization, and the protein concentration (in g/100 ml) was calculated by multiplying the figures for N by 0.25.

The albumin fraction was estimated by the same method, the globulin having been precipitated by sodium sulphite (Campbell & Hanna, 1937)

Estimation of the extracellular fluid phase of a tissue The extracellular space of a tissue can be expressed either per 100 g of fat-free solids or per 100 g of fat-free wet tissue (Darrow, 1946, Fulton, 1946) The first method gives a better correlation between changes of the fat free solids and their constituent N, P and K, on the other hand, the relationship between fat-free solids and tissue water, Cl^- and Na is not precise (Darrow, 1946) As changes of water, Cl^- and Na content of tissue were the main object of the present investigations, the second method was deliberately adopted

The extracellular fluid phase was expressed in terms of Cl^- (or Na) space The method of calculating the Cl^- space was that of Eichelberger (1939)

$$\text{Extracellular water (ml/100 g tissue)} = \frac{\text{mg } \text{Cl}^- / 100 \text{ g tissue} \times \text{plasma water content}}{\text{mg } \text{Cl}^- / 100 \text{ g plasma} \times 1.04} \times 100$$

To calculate the Na space a similar formula was used

$$\text{Extracellular water (ml/100 g tissue)} = \frac{\text{mg Na/100 g tissue} \times \text{plasma water content}}{\text{mg Na/100 g plasma} \times 0.95} \times 100$$

The cellular phase (g/100 g tissue) = 100 - extracellular fluid phase

Calculation of the intracellular fluid phase of a tissue The intracellular fluid phase was obtained by subtracting the figure for the extracellular fluid phase from that of the total amount of water in 100 g tissue

Statistical treatment Student's 't' test for small samples, as described by Fisher (1944), was used for estimation of the significance of means The probability 'P' for 't' was obtained from Fisher & Yates's (1943) tables

RESULTS

External appearance of rats on protein-deficient diets

Rats fed on *TT* diet for 7 and 14 days looked perfectly healthy, though their weight had decreased by 17 and 23 % respectively However, rats fed on *TT* diet for 6 weeks looked emaciated Their loss of

body weight amounted to 33% Their coats lost the normal smooth appearance, but the skin of the tail and feet was smooth No ophthalmia or diarrhoea was noticed

Rats fed on the low protein, high carbohydrate diet (*LCT*) lost 30 % of their weight in 70 days Nearly all of these animals showed large patches of alopecia, either on the back or on the head They did not look emaciated

Gross post mortem findings in rats fed on protein deficient diets

The post mortem findings were as follows nothing abnormal noted in rats fed on *TT* diet for 7 or 14 days Oedema of the prepuce was observed in some of the rats which had received it for 6 weeks No ascites was found in the present series About two thirds of the animals had 'moist' tissues, while the remainder seemed to be abnormally 'dry' None of the thoracic or abdominal organs showed any changes, in particular the liver appeared entirely normal The latter findings conform to those made with a previous series of *TT* rats (Dicker *et al.* 1946) No ascites was found in *LCT* rats All these animals had fatty deposits in the abdomen, their livers had a markedly fatty appearance

Changes in plasma water concentration

There was no significant decrease in the plasma solids of rats fed on *TT* diet for 7 days as compared with control rats ($t = 1.312$, $P = 0.2$), nor was there any significant change in the protein concentration of their plasma (Table 1) But after 2 weeks on *TT* diet there was a marked increase in the plasma water accompanied by a decrease in the plasma protein concentration Expressed in terms of plasma solids, there was a fall of 15%, while the protein level fell by 10 % (Table 1)

The dilution of the plasma became more apparent the longer the period of malnutrition after 6 weeks on *TT* diet the fall in the plasma solids and in the

Table 1 *Effect of protein-deficient diets on the total water content of plasma, muscle, liver and brain of rats*

(The values are means and standard errors In parentheses, number of rats All values for muscle and brain are expressed per 100 g fat-free tissue)

Treatment of rats	Plasma protein concentration (g/100 ml)	Plasma water content (g/100 ml)	Muscle water content (g/100 g)	Liver water content (g/100 g)	Brain water content (g/100 g)
Fed on a standard diet (<i>ST</i>)	7.06 ± 0.090 (27)	91.4 ± 0.13 (27)	75.7 ± 0.32 (27)	71.4 ± 0.31 (27)	83.6 ± 0.24 (11)
Fed for 7 days on a protein-deficient diet (<i>TT</i>)	6.75 ± 0.331 (9)	91.8 ± 0.15 (9)	75.0 ± 0.51 (9)	72.6 ± 0.48 (9)	82.7 ± 0.21 (9)
Fed for 14 days on a protein deficient diet (<i>TT</i>)	6.46 ± 0.213 (9)	92.7 ± 0.21 (9)	77.0 ± 0.23 (9)	72.3 ± 0.75 (9)	82.2 ± 0.12 (9)
Fed for 42 days on a protein deficient diet (<i>TT</i>)	5.02 ± 0.137 (29)	93.2 ± 0.16 (29)	77.2 ± 0.28 (30)	74.9 ± 0.39 (30)	83.3 ± 0.41 (12)
Fed for 70 days on a low casein diet (<i>LCT</i>)	4.91 ± 0.186 (6)	93.6 ± 0.16 (6)	74.2 ± 0.55 (6)	68.3 ± 1.70 (6)	82.9 ± 0.70 (6)

concentration of proteins was 21 and 27% respectively (Table 1)

The same phenomenon was noticed in rats fed on a low casein diet (*LCT*) for a longer period. In spite of the gross accumulation of fat in the body, plasma solids and proteins fell by nearly 30%

Changes in the water content of tissues

No changes in the total water content of muscles were found in animals which had been fed on *TT* diet for 7 days as compared with control rats ($t=1.136$, $P>0.2$) (Table 1). However, the liver already showed a slight increase of its total water content ($t=2.010$, $P<0.1$)

Table 1 shows changes in total water content of muscles, liver and brain in rats fed on *TT* diet for 14 and 42 days

When comparing total water content of muscles and liver of rats after 42 days of *TT* diet and after 70 days on *LCT* diet, it will be seen that the latter animals had significantly less water in their tissues than the former ($t=4.411$, $P<0.001$, and $t=5.500$, $P<0.001$), the water content of muscle and liver samples from *LCT* rats was actually comparable with that of control rats

Changes in the ionic composition of plasma and tissues

Plasma Changes in the chloride, sodium and potassium concentration of plasma in *TT* rats during varying periods of observation are shown in

Table 2. Comparing these changes with those of the plasma water (Table 1) in the same rats at corresponding periods, it will be seen that changes in the ionic pattern are independent of those of plasma water content, i.e. occasional decreases in plasma sodium (after 7 days) or chloride concentration (after 14 days) are not the result of a dilution of the plasma

The independence of the concentration of the ions in plasma from that of the plasma water emerges even more clearly when the changes in the potassium level of the plasma are considered: the potassium level in the plasma of *TT* rats rose during the whole period of observation, in spite of the rising dilution of the plasma. In the *LCT* rats, it remained unaffected

Tissues It would be tempting to assume that the fact that chloride and sodium were not diluted by an increased amount of water in plasma was the result of an influx of these ions from the tissues. However, Table 2 shows that so far as chloride and sodium are concerned, their concentration increased significantly in muscle and in liver ($t=7.529$, $P<0.001$ and $t=3.231$, $P<0.001$). This was already apparent in rats fed on a turnip diet for 7 days only.

Comparing Tables 1 and 2, it will be seen that, as in plasma, changes in the chloride and sodium concentration of muscle and liver were independent of the concurrent changes in their water content. But in contrast to that of muscle and liver tissues, the

Table 2 Ionic composition of plasma, muscle, liver and brain in normal and hypoproteinaemic rats

(The values are means and standard errors. In parentheses, number of rats. All values for muscle and brain are expressed per 100 g fat-free tissue.)

Treatment of rats	Cl ⁻ (m equiv /l water)	Na (m equiv /l water)	K (m-equiv /l water)	Cl ⁻ (m equiv /l water)	Na (m equiv /l water)	K (m equiv /l water)
	Plasma			Muscle		
Fed on a standard diet (<i>ST</i>)	83.2±2.34 (27)	128.3±7.05 (18)	5.6±0.20 (17)	20.2±0.47 (17)	28.8±1.48 (17)	126.2±7.73 (17)
Fed for 7 days on a protein deficient diet (<i>TT</i>)	84.6±3.08 (9)	119.3±7.53 (9)	5.0±0.27 (9)	27.8±0.82 (9)	35.2±3.59 (9)	107.6±5.78 (9)
Fed for 14 days on a protein deficient diet (<i>TT</i>)	83.7±4.90 (9)	124.0±6.94 (9)	6.0±0.25 (9)	31.5±1.01 (9)	46.5±1.42 (9)	112.4±7.14 (9)
Fed for 42 days on a protein deficient diet (<i>TT</i>)	85.9±2.77 (29)	108.3±2.12 (11)	7.1±0.58 (11)	46.7±2.44 (29)	69.5±4.22 (11)	96.6±3.84 (11)
Fed for 70 days on a low casein diet (<i>LCT</i>)	81.0±2.03 (6)	120.6±2.46 (6)	5.1±0.20 (6)	37.2±2.09 (6)	60.0±4.60 (6)	107.9±5.30 (6)
	Liver			Brain		
Fed on a standard diet (<i>ST</i>)	33.6±2.80 (11)	31.0±2.09 (10)	112.8±7.63 (10)	42.7±2.89 (9)	49.0±3.56 (10)	124.0±10.51 (10)
Fed for 7 days on a protein deficient diet (<i>TT</i>)	41.4±2.99 (9)	50.7±5.61 (9)	103.7±4.87 (9)	41.1±2.69 (9)	52.3±4.37 (9)	129.5±5.26 (9)
Fed for 14 days on a protein deficient diet (<i>TT</i>)	40.2±0.66 (9)	60.6±3.00 (9)	97.8±8.13 (9)	38.0±1.75 (9)	56.5±2.42 (9)	118.6±3.08 (9)
Fed for 42 days on a protein deficient diet (<i>TT</i>)	36.4±2.55 (11)	48.7±3.24 (11)	93.8±3.61 (11)	39.1±3.27 (11)	50.5±3.25 (11)	114.4±4.44 (11)
Fed for 70 days on a low casein diet (<i>LCT</i>)	49.0±2.69 (6)	57.2±6.21 (6)	120.0±6.63 (6)	41.7±1.25 (6)	58.4±3.36 (6)	109.7±8.89 (6)

ionic composition of brain samples remained constant during the whole period of observation (Table 2)

Such changes in the water content and in the ionic composition of plasma and tissues made it likely that variations had occurred in the amount of extracellular water in muscle and liver

Extracellular-fluid phase of tissues

In normal rats fed on a standard diet (*ST*) the chloride space of muscle, liver or brain was comparable with, and almost equal to, the sodium space (Table 3). It will also be seen from Table 3 that the extracellular-fluid phase was of characteristic magnitude in each of the tissues investigated and showed little variation in individual normal animals.

However, in *TT* rats, the extracellular fluid phase of muscle and liver was increased. This increase was already significant after 7 days: it amounted to 44% in muscles and to 50% in liver. It increased steadily. The increase after 6 weeks amounted to 163 and 55% of the normal.

In Table 3 all data obtained for rats kept on *TT* diet for 42 days were pooled, irrespective of the fact that about one third of these animals had particularly dry and thin muscles. But the individual figures obtained from analysis of muscle samples of such 'dry' animals showed a marked increase in the extracellular-fluid phase, amounting to nearly 140%. Whether 'dry' or 'wet', all the *TT* rats had therefore a marked 'oedema' of their tissues. It follows that there was no essential difference between the two types of protein-deficient animals (Dicker, 1947).

In rats fed on a low casein, high carbohydrate diet (*LCT*) the extracellular fluid phase of muscle and liver was also increased. The increase amounted to 120 and 70% respectively. It is thus clear that the increased extracellular-fluid phase of *TT* and *LCT* rats was not the result of a deficiency of calories.

It could thus be shown that there were signs of early oedema in the tissues of rats fed on a protein-deficient diet. This increase of the extracellular fluid space developed in spite of normal plasma protein

Table 3 *Extracellular- and intracellular-fluid phases (chloride and sodium) of tissues of normal and hypoproteinaemic rats*

(The values are means and standard errors. In parentheses, number of rats. All values for muscle and brain are expressed per 100 g fat-free tissue.)

Treatment of rats	Extracellular fluid phase (ml/100 g)		Intracellular fluid phase (ml/100 g)	
	Cl ⁻	Na	Cl ⁻	Na
Muscle				
Fed on a standard diet (<i>ST</i>)	16.7±0.50 (27)	16.8±1.03 (17)	59.0±0.50 (27)	58.8±1.11 (17)
Fed for 7 days on a protein-deficient diet (<i>TT</i>)	24.1±1.54 (9)	28.1±3.68 (9)	50.8±1.74 (9)	46.9±3.94 (9)
Fed for 14 days on a protein-deficient diet (<i>TT</i>)	32.3±2.78 (9)	30.1±1.89 (9)	44.7±2.72 (9)	47.0±1.83 (9)
Fed for 42 days on a protein-deficient diet (<i>TT</i>)	44.8±1.90 (29)	43.5±2.08 (11)	32.4±1.64 (29)	33.6±0.70 (11)
Fed for 70 days on a low casein diet (<i>LCT</i>)	36.9±3.63 (6)	36.8±2.01 (6)	37.3±3.65 (6)	37.4±2.27 (6)
Liver				
Fed on a standard diet (<i>ST</i>)	22.6±2.50 (11)	21.3±1.55 (10)	48.5±2.05 (11)	50.1±2.03 (10)
Fed for 7 days on a protein-deficient diet (<i>TT</i>)	34.3±2.64 (9)	35.2±2.23 (9)	38.6±2.97 (9)	37.6±2.94 (9)
Fed for 14 days on a protein-deficient diet (<i>TT</i>)	38.1±2.56 (9)	34.8±2.73 (9)	34.2±2.57 (9)	48.0±2.74 (9)
Fed for 42 days on a protein-deficient diet (<i>TT</i>)	35.4±2.66 (11)	32.6±1.91 (11)	39.5±2.52 (11)	42.3±2.14 (11)
Fed for 70 days on a low casein diet (<i>LCT</i>)	39.0±2.03 (6)	35.4±3.22 (6)	29.3±2.07 (6)	32.8±4.09 (6)
Brain				
Fed on a standard diet (<i>ST</i>)	38.6±3.31 (9)	40.1±3.00 (10)	44.9±3.24 (9)	43.5±2.89 (10)
Fed for 7 days on a protein-deficient diet (<i>TT</i>)	38.7±2.62 (9)	43.0±4.82 (9)	43.2±2.81 (9)	39.5±4.90 (9)
Fed for 14 days on a protein-deficient diet (<i>TT</i>)	40.6±2.24 (9)	38.1±2.24 (9)	41.8±2.18 (9)	44.0±2.29 (9)
Fed for 42 days on a protein-deficient diet (<i>TT</i>)	40.4±3.31 (11)	43.9±2.71 (11)	42.9±3.60 (11)	39.4±2.37 (11)
Fed for 70 days on a low casein diet (<i>LCT</i>)	41.0±1.51 (6)	38.4±2.14 (6)	41.9±1.40 (6)	44.9±2.31 (6)

concentrations. It could be shown further that the extracellular fluid space of tissues (muscle and liver) increased whether the protein deficient diet was, or was not, lacking in calories.

The intracellular fluid phase of tissues

Can the increased extracellular fluid phase observed in the different series of *TT* and *LCT* rats be explained by changes in the total muscle water? Comparing the data of Table 1 and Table 3, it could be concluded that this was not the case. In animals fed on *TT* diet for 7 days the extracellular fluid space increased without any changes in the total muscle water. Even in rats fed on *TT* diet for 14 or 42 days the increase in the total water of the tissues (Table 1) was far less pronounced than that of the extracellular fluid space (Table 3). It follows that tissue oedema developed concurrently with a marked loss of intracellular water. This is even clearer with *LCT* rats (Table 3).

It is thus clear that the retention of tissue water resulting from a lack of protein in the food did not produce a swelling of the tissue cells, on the contrary, protein deficiency led to a decrease of intracellular

water. This decrease became the more obvious the more the extracellular fluid space increased.

Colloid osmotic pressure of plasma

It has not been shown so far whether the decreases in the plasma protein concentration observed led to corresponding decreases in plasma colloid osmotic pressure. Values of 18.9 ± 0.12 cm water, corrected to a temperature of 37° , were obtained for normal rat plasma. These values were somewhat lower than those found by Landis (1930), but this difference may be accounted for by the difference in pH and in electrolyte concentration of the buffer solution producing different Donnan effects (Marack & Hewitt, 1927, Keys & Taylor, 1935a).

Table 4 gives figures for the colloid osmotic pressure of plasma samples from normal and hypoproteinaemic rats.

Dilutions of plasma of normal rats were made by adding varying amounts of the buffer solution. Fig. 1 shows that the colloid osmotic pressure of progressive dilutions of plasma of normal rats yielded values which form a straight line when plotted against the plasma protein concentration.

Table 4 Colloid osmotic pressure of plasma samples from normal and hypoproteinaemic rats

	Plasma proteins (Kjeldahl) (g/100 ml)	Albumin/globulin ratio (A/G)	Plasma colloid osmotic pressure (cm water)
Normal rats	7.48	1.20	19.8
	7.48	1.10	19.6
	7.25	1.20	20.0
	7.04	1.17	19.4
	6.75	1.07	19.2
Mean and s.e.	7.20 ± 0.100	1.15 ± 0.023	19.6 ± 0.12
Rats fed on a protein-deficient diet (<i>TT</i>) for 7 days	7.18	0.98	19.1
	7.08	1.00	19.6
	7.00	1.26	19.4
	6.87	0.95	18.5
Mean and s.e.	7.03 ± 0.057	1.05 ± 0.031	19.2 ± 0.10
Rats fed on a protein deficient diet (<i>TT</i>) for 14 days	6.03	0.83	17.7
	6.55	0.90	17.3
	6.08	0.80	16.3
	6.07	0.70	15.4
	6.00	0.70	14.6
Mean and s.e.	6.22 ± 0.121	0.78 ± 0.011	16.3 ± 0.51
Rats fed on a protein deficient diet (<i>TT</i>) for 42 days	5.94	0.60	12.8
	5.94	0.60	10.8
	5.45	0.68	13.7
	5.27	0.50	8.6
	5.16	0.60	10.0
	5.10	0.62	11.7
	4.52	0.50	5.5
	3.95	0.50	8.6
Mean and s.e.	5.16 ± 0.223	0.57 ± 0.007	10.2 ± 0.87
Rats fed on a protein-deficient diet (<i>LCT</i>) for 70 days	5.14	0.56	10.6
	4.37	0.67	10.8
	4.37	0.68	12.7
	3.91	0.68	11.8
	3.86	0.59	7.8
Mean and s.e.	4.33 ± 0.205	0.63 ± 0.023	10.6 ± 0.71

This relationship applied only for dilutions of the plasma up to 50% of the initial concentration, confirming Verney's (1926) findings on human plasma

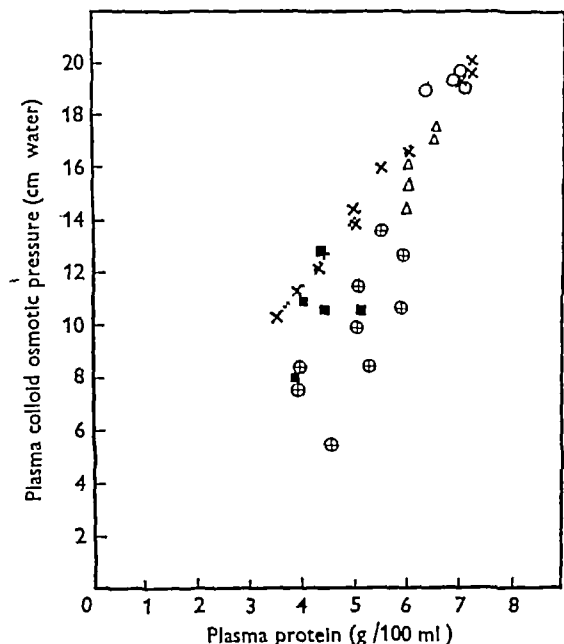


Fig 1 Relation between the plasma colloid osmotic pressure and plasma protein concentration. x = normal rats, O = rats fed for 7 days on a vegetable protein deficient diet, Δ = rats fed for 14 days on a vegetable protein deficient diet, ⊕ = rats fed for 42 days on a vegetable protein deficient diet, ■ = rats fed for 70 days on a low casein diet. All the values for colloid osmotic pressure are corrected to 37°

Comparing the values for the colloid osmotic pressure of diluted normal plasmas with those of samples from hypoproteinaemic rats, it will be seen (Fig 1) that, at the same concentration of plasma proteins, the colloid osmotic pressure of plasma of rats fed on *TT* diet for 42 days and of *LCT* rats was lower than that of the normal animals. This decrease in the colloid osmotic pressure per g plasma protein in animals fed on a low protein diet was progressive. Plasmas of rats fed on *TT* diet for 7 days did not show a significant fall in the colloid osmotic pressure ($t = 1.581$, $P > 0.1$), but after 14 days the fall of colloid osmotic pressure was greater than that produced by a corresponding dilution of plasma of normal rats. It is thus clear that the low figures obtained for the osmotic pressure of plasma proteins in rats fed on protein deficient diets could not be the result of a simple dilution of the blood of these animals.

The decrease in the colloid osmotic pressure observed in the plasma of rats fed on protein deficient diets was closely correlated with the fall in the concentration of albumin, that of the globulin

remained more or less unaffected. In other words, the colloid osmotic pressure per g of total plasma proteins was directly proportional to the albumin/globulin (A/G) ratio (Table 4). However, in a few cases plasma samples with the same concentration of proteins had a widely different colloid osmotic pressure, e.g. two plasma samples with protein concentrations of 5.9 g/100 ml gave values for the colloid osmotic pressure of 12.8 and 10.8 cm water. The difference in these instances could not be accounted for by changes in the albumin/globulin ratio (A/G = 0.60 in both cases). Two other samples with the same A/G ratio (0.50) had a colloid osmotic pressure of 8.6 and 5.5 cm water respectively. It is thus clear that in these particular cases, changes in the A/G ratio did not account for the low colloid osmotic pressure. It thus seems likely that the plasma proteins of some of the hypoproteinaemic animals underwent other changes in chemical or physical composition besides the decrease in the albumin fraction.

Finally, is there a relation between the colloid osmotic pressure of plasma and the occurrence of tissue oedema? Comparing the figures in Table 3 and Table 4 it will be seen that the increase in the extracellular fluid space in rats fed on *TT* diet for 7 days occurred in animals with normal plasma protein concentration, normal A/G ratio and normal colloid osmotic pressure. Furthermore, no correlation could be found between the size of the extracellular fluid space and the plasma colloid osmotic pressure.

DISCUSSION

There is ample evidence that chloride and sodium in rat and dog muscle are mainly extracellular (Fenn, 1936, Harrison, Darrow & Yarnet, 1936, Hastings & Eichelberger, 1937, Manery & Bale, 1941). Further proof for the validity of this statement comes from the work of Manery & Haege (1941), Hevesy & Rebbe (1940) and Cuthbertson & Greenberg (1945) with radioactive isotopes ^{38}Cl and ^{24}Na (or ^{22}Na). These authors have shown that these isotopes were extracellular and that their distribution was the same. Finally, there is a satisfactory agreement between values for chloride space in muscle and liver and the extracellular space as determined microscopically in frozen sections of these tissues (Höber, 1947, Truax, 1939). At the same time it must be remembered that chloride and sodium spaces are likely to be somewhat larger than the true extracellular fluid space, even in normal animals (Fulton, 1946), and that the possibility obtains that pathological conditions may produce further alteration of cell permeability. The chloride and sodium spaces may, therefore, not be quite identical with the true extracellular space. The term extracellular fluid space in this paper must be understood to refer to

the apparent volume distribution of chloride and sodium.

In the present investigation it could be shown that, for muscles of normal rats, there is good agreement between the values of the extracellular fluid phase, whether expressed in terms of chloride or of sodium space ($t=0.113$, $P>0.9$). The values compare favourably with those found by means of radio active isotopes in the muscle of rats (Cuthbertson & Greenberg, 1945) and in dogs (Eichelberger, 1941). In the liver also it could be shown that the values for extracellular fluid phase, whether expressed as chloride or as sodium space, were comparable ($t=0.429$, $P>0.6$). They were of the same magnitude as that of chloride space of liver found in dogs (Eichelberger, 1941).

When defining the intracellular fluid phase it must be remembered that the amount of water inside the cell is correlated with the amount of potassium and that, under normal conditions, there is no shift of bases across the cell membrane (Peters, 1944). There is a reciprocal relationship between the extracellular sodium and the intracellular potassium, in other words, the amount of intracellular potassium, and intracellular water, is indirectly influenced by the concentration of extracellular sodium (Darrow, 1945).

It could be shown in the present investigation that the extracellular fluid phase of muscle of normal adult rats was fairly constant. However, in rats fed on a protein-deficient diet for a short period only (7 days) the extracellular fluid space of muscle increased significantly. This onset of tissue oedema occurred without significant changes in the plasma water content, the plasma protein concentration or the osmotic pressure of the plasma proteins. These findings agree with those of Keys *et al.* (1946) in human beings, receiving experimentally a low protein diet, in whom an increase of the thiocyanate space (i.e. of the extracellular space) could be demonstrated at a time when the plasma protein concentrations, the plasma electrophoretic pattern and the plasma colloid osmotic pressure were still normal. It thus seems difficult to attribute the early onset of the oedema to a fall of the colloid osmotic pressure (Starling's hypothesis), but it might still be due to an increase in the capillary pressure (Pappenheimer & Soto Riviera, 1947). It was not possible to estimate the venous pressure in the present series of rats, but in human beings Keys *et al.* (1946) showed that the venous blood pressure had fallen by nearly 50% of the normal value.

The cause of the early increase in the extracellular fluid phase must thus be sought elsewhere. The rats fed on a vegetable low protein diet were lacking in both proteins and calories. There are thus good reasons to assume that a cellular breakdown was accelerated. Hence the early tissue oedema may

have been due to an accumulation of waste products if of small molecular weight they would exert a high osmotic pressure. The same process may also involve liberation of potassium ions, which in normal tissue were osmotically inactive. A higher osmotic pressure within the cell would result in an increased concentration of chloride and sodium in the extracellular fluid space in an attempt to equalize osmotic pressure. To be able to maintain a balance between forces engaged in such a dynamic equilibrium the cells would extrude their waste products and with them part of their potassium, and ultimately would lose some of the intracellular water. This assumption would agree with the findings of Gamble, Ross & Tisdall (1923), who showed that starvation induces a loss of potassium from the body corresponding to the potassium content of the muscle tissue lost.

The data in Table 3 show that the increase of the extracellular fluid phase was significantly greater than the decrease of the intracellular water. This can only mean that the increased extracellular fluid phase was made up mainly by water from the plasma. After 7 days on a low protein, low calorie diet (*TT*) the plasma water content and its protein concentration were normal (Table 1). This is in agreement with findings in dogs fasting for a period of 4-6 days (Kerr, Hurwitz & Whipple, 1918). The unchanged level of the plasma proteins in rats fed for 7 days on *TT* diet might be explained by changes in the plasma volume masking the loss of plasma proteins, resulting from a state of semi starvation. It would also agree with the findings of Metcalf *et al.* (1945) in rats kept on a protein deficient diet.

At a later stage, in rats fed on *TT* diet for 14 days, the concentration of plasma proteins was decreased, though the A/G ratio was not significantly altered, and the colloid osmotic pressure was somewhat lower than in controls (Table 4, Fig. 1). Though small, these changes in the colloid osmotic pressure of plasma may have been sufficient to produce an increase of the extracellular fluid phase, provided that there was no concurrent drop in the capillary pressure.

In rats fed on *TT* or *LCT* diet for several weeks the colloid osmotic pressure of the plasma proteins was markedly lower than that of the controls. In some cases it was even lower than that expected for the corresponding amount of plasma proteins. Such discrepancies between the plasma protein concentration and the expected colloid osmotic pressure have been reported in patients suffering from malnutrition (Govaerts & Legume, 1942) and in young men exercised to exhaustion where, in spite of a marked increase (averaging 12.5%) in serum protein concentration, the colloid osmotic pressure decreased (Keys & Taylor, 1935b). In rats fed on a protein-deficient diet (*TT* or *LCT*) the globulin fraction did not seem to be seriously affected, it is thus likely that the abnormally low colloid osmotic

pressure encountered in some cases was the result of changes in the albumin fraction. Two explanations of this phenomenon may be proposed.

(a) The chemical analysis of plasma proteins after precipitation of globulins by sodium sulphite may have given too high values for the albumin fraction (Cohn & Wolfson, 1947). This hypothesis receives further support from the work of Zeldis, Alling, McCoord & Kulka (1945). Comparing data obtained by chemical analysis and by electrophoretic methods these authors have shown that in dogs fed on a protein deficient diet for 12 weeks the degree of depletion of 'electrophoretic albumin' is considerably greater than that of the chemical albumin fraction.

(b) Changes in the molecular dimension of the proteins or in their ionization, or in both, provide the basis for another interpretation. For any change in the colloid osmotic pressure to be due to changes other than in the molecular dimension, there must be changes in the ionization of the proteins or changes in the concentration of the crystalloids of the medium (Keys & Taylor, 1935*b*). Both pH and ionic strength, inside and outside the collodion membrane of the osmometer, remained constant in the present experiments. This makes any appreciable change in the magnitude of the Donnan effect unlikely. Furthermore, it has been shown that the colloid osmotic pressure of blood plasma is little affected by changes in pH (Marrack & Hewitt, 1927) or by moderate changes in salt concentration (Meyer, 1932).

It would seem that there are two stages in the production of oedema in tissue resulting from feeding rats on a protein deficient diet. It could be shown that a significant increase of the extracellular-fluid phase of muscles occurred very early, without any significant changes in the water content or the protein concentration of plasma, no changes in the colloid osmotic pressure of plasma were observed at this stage. It must therefore be concluded that the onset of oedema formation in malnutrition is independent of changes in the colloid osmotic pressure of plasma. At a later stage further increases of the extracellular-fluid phase coincided with the progressive fall in the colloid osmotic pressure of the plasma. However, even then no clear correlation could be found between the degree of hypoproteinaemia, the fall of the colloid osmotic pressure of plasma and the magnitude of the extracellular fluid phase of tissues.

SUMMARY

1 The total water content of plasma, of skeletal muscle, of liver and of brain was estimated in normal rats and in rats fed for periods of 7, 14 and 42 days on a protein deficient diet. Extracellular- and intracellular-fluid phases of these tissues were calculated by means of chloride and sodium estimations.

2 The calculated values for the extracellular fluid phase of these tissues agreed well, whether expressed as chloride or as sodium space.

3 In normal rats fed on a standard diet (*ST*) the extracellular fluid phase of muscle, liver and brain, expressed as chloride space, amounted to 16.7 ± 0.50 , 22.6 ± 2.50 and 38.6 ± 3.31 ml/100 g fat-free tissue respectively.

4 In rats fed for 7 days on a vegetable protein deficient diet (*TT*) the water content of plasma and of tissues, the plasma protein concentration and the plasma colloid osmotic pressure remained unchanged. In spite of this the extracellular-fluid phase of skeletal muscles and of liver had significantly increased, showing that there was tissue oedema with normal plasma protein concentration and normal colloid osmotic pressure.

5 In rats fed for 14 days on a vegetable protein deficient diet (*TT*) there was a significant increase in the water content of the plasma, the muscles and the liver, with a decrease in the plasma protein concentration and a slight but significant decrease in the colloid osmotic pressure of plasma. The extracellular-fluid phase of the muscles was significantly greater than that of rats fed for 7 days on *TT* diet.

6 Comparing data obtained for rats fed for 42 days on a low-protein low calorie diet (*TT*) with those for animals fed for 70 days on a diet equally low in protein but yielding an adequate supply of calories (*LCT*) it could be shown that the extracellular fluid phases of muscle and liver were markedly increased, and the concentration of proteins in plasma significantly decreased in both series.

7 The mean colloid osmotic pressure of normal rat plasma amounted at 37° to 19.6 ± 0.12 cm water, that of rats fed on *TT* diet for 7 days was 19.2 ± 0.19 cm, that of rats fed on *TT* diet for 14 days was 16.3 ± 0.51 cm, that of animals fed on *TT* diet for 42 days was 10.2 ± 0.87 cm, and that of animals fed on *LCT* diet for 70 days amounted to 10.6 ± 0.71 cm water.

8 The colloid osmotic pressure of plasma of the three latter groups was correlated with the albumin/globulin (A/G) ratio of plasma, but not with the nitrogen concentration of the plasma samples.

9 In the cases of a few rats fed for 42 days on *TT* diet and for 70 days on *LCT* diet, the colloid osmotic pressure of plasma samples was lower than that expected from either the A/G ratio or the plasma nitrogen concentration.

10 No correlation could be found between the increase of the extracellular fluid phase of tissues and the decrease of the colloid osmotic pressure of the plasma.

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Changes in the Extracellular- and Intracellular-Fluid Phases of Tissues during Water Diuresis in Normal and Hypoproteinaemic Rats

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It has been shown in a previous paper (Dicker, Heller & Hower, 1946) that the urinary excretion of administered water by protein deficient rats is delayed, and that the total amount of water excreted in 3 hr is lower than that of normal animals. There would seem to be at least three likely explanations for the abnormal and delayed diuretic response observed in these rats: (a) a slower rate of water absorption from the alimentary canal, (b) an increased 'preparedness' of extrarenal tissues to retain

water, resulting in a decrease of the plasma water load, (c) a failure of the renal tubules to reduce the rate of water reabsorption as a response to the increased water load, or a combination of these factors.

To investigate these possibilities, the rate of water absorption from the gastrointestinal tract was estimated according to Heller & Smirk (1932), and the partition of absorbed water between the extracellular fluid phases of tissues was determined.

METHODS

Experimental animals Adult male albino rats weighing 265–310 g were used.

Diets The standard diet (*ST*) and the vegetable low protein diet (*TT*) conformed to the description given in the previous paper (Dicker, 1948*a*)

Procedure Normal rats were fed on a standard diet (*ST*) for several weeks. Food, but not water, was withheld 24 hr before the diuresis experiments. The animals were then given water to the extent of 5% of their body weight by stomach tube, and were killed 15, 30, 45, 60, 75, 90 and 120 min after the administration.

Another series of rats was fed on diet *TT*. After 6 weeks on this diet, food was withheld for 24 hr and water to the extent of 5% of their body weight was given. They were killed 15, 30, 45, 60 and 90 min after the administration.

Immediately before they were due to be killed, the rats were anaesthetized and blood collected from the carotid and jugular was mixed with heparin. Immediately after death the cardia and the distal end of the small intestine (excluding the caecum) were ligatured. The alimentary canal was weighed with its contents. Muscle, liver and brain samples were obtained and the amount of water, Cl^- , Na and K in these tissues estimated.

Chemical methods Cl^- , Na, K and water estimations in plasma and tissue samples conformed to those described previously (Dicker, 1948*a*). Water content was calculated for fat-free tissues.

Protein concentration in plasma was estimated by two different methods: (a) a micro Kjeldahl method, (b) the copper sulphate method for measuring plasma specific gravities (Phillips, Van Slyke, Dole, Emerson, Hamilton & Archibald, 1945). The formula used for the calculation of the plasma protein concentration was that proposed by Hoch & Marrack (1945). The two methods gave results which were not significantly different (Dicker, 1948*b*).

Estimation of the extracellular and intracellular fluid phase of tissues Extracellular and intracellular tissue fluid phases were calculated from estimation of Cl^- and Na spaces, as in the previous paper (Dicker, 1948*a*), where the identity of these spaces with the true extracellular-fluid phase has also been discussed.

Sampling of rats The rats were killed in groups consisting of animals with varying absorption times. For statistical treatment of the data, results obtained for animals with identical absorption times were pooled.

Statistical treatment Results are given as mean and standard errors. Student's *t* test for small sample method was used for the significance of means and calculated according to Mainland (1938). *P* values for *t* were obtained from Fisher & Yates's (1943) tables.

RESULTS

Gross post-mortem observation

Rats fed on diet *TT* for 6 weeks had lost about 35% of their body weight, the concentration of proteins in plasma had equally decreased by 35%. No ascites or free fluid was found in any of these animals, even after administration of water, but nearly all those that had received water had abnormally 'wet' tissues. The perirenal and retro-

peritoneal connective tissues were particularly oedematous, and had a gelatinous appearance. Pressure on these tissues released a substantial quantity of water (up to 1.5 ml).

In all other respects, these series of protein deficient animals were comparable with those described in the previous paper (Dicker, 1948*a*).

Water absorption

Fig 1 shows the rate of water absorption from the alimentary canal in normal and hypoproteinaemic rats. The percentage of body weight of the alimentary canal in normal control rats was 3.6 as compared with 5.7 in controls fed on *TT* diet. In order to render the two curves more easily comparable 2.1 (i.e. the difference between the weights of the alimentary canal in the two series) was deducted from the mean values obtained in the protein-deficient series (Fig 1).

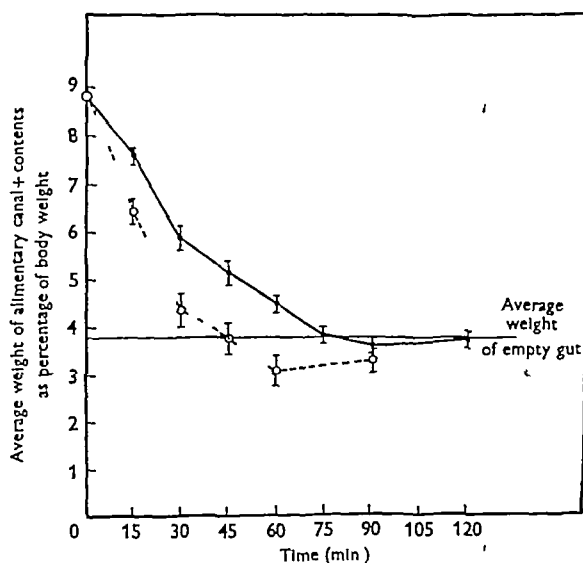


Fig 1 Average water absorption curves of rats (a) ●—● in normal rats, (b) ○---○ in hypoproteinaemic rats. The average weight of the empty gastro intestinal tract was 3.6% of the body weight in normal control rats as compared with 5.7% of the body weight in controls fed on *TT* diet. In order to render the two curves comparable 2.1% was deducted from the mean values obtained in the protein deficient series. 5% of the body weight of water was given making 8.6% the starting point of the absorption curves. The vertical lines represent the standard error.

In normal rats the absorption of 5.0% of body weight of water by the alimentary canal was practically finished in 60–75 min. This is in agreement with Heller & Smirk's (1932) findings. In protein deficient rats the absorption of the standard amount of water was terminated in 45–60 min (Fig 1).

It is thus quite clear that the rate of water absorption from the gut in hypoproteinaemic rats is not slower than that in normal rats, and cannot therefore be the cause of the delayed onset of the water diuresis in these animals

Changes in water concentration of plasma and tissues in normal and hypoproteinaemic rats

Changes in the water concentration of plasma and tissues after water administration, in both normal and hypoproteinaemic rats, are shown in Table 1. In normal rats the absorption of water resulted in an

in the plasma protein concentration (Table 1). It would seem that the water left the blood plasma at approximately the same rate as it was absorbed from the alimentary canal.

In normal rats the amount of water in muscle and liver was significantly increased 30 and 45 min after water administration ($t=3.107$, $P<0.001$, and $t=3.779$, $P<0.001$, respectively). The maximum increase in the water load of these tissues occurred at about 60 min.

In protein deficient rats (*TT*) no significant changes in the amount of water in the liver could be

Table 1 *The effect of water administration on the water content of plasma, muscle, liver and brain of normal and of hypoproteinaemic rats*

(The values are means and standard errors. Numbers of rats in parentheses)

Stage of observation	Plasma water content (%)	Muscle water content (%)	Liver water content (%)	Brain water content (%)	Plasma proteins (Kjeldahl) (g/100 ml)
Normal rats					
Before water administration	91.4±0.13 (27)	75.8±0.32 (27)	71.4±0.31 (27)	83.6±0.24 (11)	7.06±0.092 (27)
15 min. after water administration	91.9±0.11 (20)	76.9±0.30 (20)	71.5±0.44 (20)	83.0±0.26 (9)	6.66±0.120 (18)
30 min. after water administration	92.3±0.10 (18)	77.0±0.15 (18)	72.0±0.33 (18)	83.9±0.34 (7)	6.54±0.182 (18)
45 min. after water administration	93.0±0.18 (18)	77.4±0.16 (18)	72.5±0.32 (18)	83.7±0.27 (7)	6.48±0.120 (18)
60 min. after water administration	92.5±0.26 (16)	77.8±0.21 (16)	72.3±0.32 (16)	84.3±0.76 (6)	6.49±0.108 (16)
75 min. after water administration	92.5±0.17 (16)	77.4±0.11 (16)	71.7±0.27 (16)	84.0±0.48 (6)	6.48±0.058 (16)
90 min. after water administration	92.6±0.13 (12)	76.8±0.13 (12)	72.2±0.30 (12)	83.9±0.31 (6)	6.48±0.075 (12)
120 min. after water administration	92.0±0.09 (12)	76.3±0.23 (12)	71.8±0.30 (12)	83.4±0.15 (6)	6.64±0.190 (12)
Hypoproteinaemic rats					
Before water administration	93.2±0.16 (29)	77.2±0.28 (30)	74.9±0.39 (30)	83.3±0.41 (12)	5.12±0.137 (29)
15 min. after water administration	93.5±0.14 (10)	77.5±0.21 (10)	74.8±0.54 (10)	83.8±0.67 (10)	4.66±0.101 (10)
30 min. after water administration	93.5±0.16 (12)	77.1±0.82 (12)	74.6±0.60 (12)	83.5±0.26 (8)	4.75±0.130 (10)
45 min. after water administration	93.7±0.16 (16)	77.6±0.50 (16)	74.1±0.48 (16)	83.5±0.33 (6)	4.73±0.163 (16)
60 min. after water administration	93.7±0.14 (20)	78.4±0.28 (20)	75.0±0.34 (20)	83.4±0.92 (8)	4.86±0.136 (20)
75 min. after water administration	—	—	—	—	—
90 min. after water administration	93.3±0.20 (7)	78.2±0.22 (7)	75.4±0.57 (7)	83.4±0.18 (7)	4.93±0.131 (7)
120 min. after water administration	—	—	—	—	—

early increase of the plasma water content ($t=2.666$, $P<0.02$). This increase reached its maximum at about 45 min. and was accompanied by a fall in the plasma protein concentration (Table 1). In hypoproteinaemic rats no significant changes in the plasma water could be found during the 90 min. of observation, nor were there any significant changes

noticed during the whole period of observation. The increase in the amount of water in muscle was delayed; the first significant increase occurred 60 min. after water administration ($t=2.120$, $P<0.05$) as compared with 30 min. in normal rats.

No significant changes in the amount of water in brain were noted in either *ST* or *TT* rats.

Changes in the concentration of electrolytes of plasma and tissues in normal and hypoproteinaemic rats

The concentration of chloride in plasma decreased soon after water administration in normal and hypoproteinaemic rats (Tables 2 and 3). The decrease in the plasma chloride concentration lasted for 30 min in normal rats, and more than 60 min in *TT* rats, in other words, it covered in both series the period of time elapsing between that of administration of water and that of the onset of water diuresis. The decrease of chloride concentration in the plasma was not the result of a dilution of the plasma. This is seen from the results obtained on rats fed on *TT* diet (Table 1) where the administration of water by stomach tube did not produce an increase of the

plasma water content. Nor can the decrease be explained by an escape of chloride into the muscles or the liver (Tables 2 and 3) in both normal and protein deficient rats: the fall in the plasma chloride concentration was concurrent with a fall in the concentration of chloride in the muscle, while the chloride concentration in the liver remained unaffected. The fall in muscle chloride in the two series of rats lasted as long as that in the plasma. Finally, it will be noted (Tables 2 and 3) that variations in plasma chloride were independent of variations in the concentrations of sodium and potassium in the plasma. These findings agree with those of Priestley (1916), Smirk (1932) and Eggleton (1937) which suggest that water diuresis is preceded by passage of chloride into the gut.

Table 2 *The effect of water administration on the concentration of electrolytes of plasma and tissues in normal rats*

(The values are means and standard errors. Number of rats in parentheses.)

Stage of observation	Cl ⁻ (m equiv /l water)	Na (m equiv /l water)	K (m equiv /l water)	Cl ⁻ (m equiv /l water)	Na (m equiv /l water)	K (m equiv /l water)
	Plasma			Muscle		
Before water administration	83.2 ± 2.34 (27)	128.3 ± 7.05 (18)	5.6 ± 0.29 (17)	20.2 ± 0.47 (27)	29.0 ± 1.48 (17)	156.2 ± 7.73 (17)
15 min after water administration	72.3 ± 1.63 (20)	112.8 ± 4.04 (10)	4.5 ± 0.31 (9)	17.5 ± 0.67 (20)	25.0 ± 2.27 (9)	155.0 ± 4.57 (9)
30 min after water administration	69.3 ± 2.04 (18)	112.3 ± 4.56 (12)	5.2 ± 0.41 (7)	18.9 ± 0.99 (18)	26.0 ± 2.73 (11)	145.3 ± 2.87 (7)
45 min after water administration	82.2 ± 2.80 (18)	134.2 ± 5.32 (15)	6.8 ± 0.66 (13)	22.5 ± 0.92 (18)	36.1 ± 1.69 (13)	134.2 ± 5.62 (13)
60 min after water administration	82.3 ± 5.54 (16)	137.5 ± 3.90 (12)	5.7 ± 0.44 (12)	22.4 ± 1.23 (16)	36.1 ± 1.94 (12)	147.2 ± 4.41 (12)
75 min after water administration	86.5 ± 2.16 (16)	137.6 ± 5.57 (16)	5.2 ± 0.26 (16)	23.2 ± 0.66 (16)	37.5 ± 1.98 (16)	158.7 ± 4.60 (16)
90 min after water administration	78.6 ± 2.62 (12)	128.3 ± 3.09 (12)	6.1 ± 0.40 (12)	20.4 ± 0.74 (12)	32.5 ± 1.12 (12)	154.0 ± 8.01 (12)
120 min after water administration	84.0 ± 1.87 (12)	131.3 ± 4.00 (12)	5.0 ± 0.26 (12)	20.7 ± 0.75 (12)	29.1 ± 1.55 (12)	157.5 ± 3.67 (12)
	Liver			Brain		
Before water administration	33.6 ± 2.80 (11)	31.0 ± 2.09 (10)	97.8 ± 7.63 (10)	42.7 ± 2.89 (9)	49.0 ± 3.56 (10)	124.0 ± 10.50 (10)
15 min after water administration	29.0 ± 2.74 (9)	38.1 ± 3.71 (9)	98.0 ± 4.56 (9)	35.3 ± 3.56 (8)	46.1 ± 4.13 (8)	137.8 ± 8.17 (9)
30 min after water administration	34.0 ± 3.83 (7)	45.5 ± 4.12 (7)	94.9 ± 4.90 (7)	33.4 ± 3.37 (7)	46.1 ± 6.20 (7)	132.7 ± 12.20 (7)
45 min after water administration	47.0 ± 1.83 (7)	55.6 ± 1.72 (7)	106.0 ± 9.36 (7)	39.0 ± 2.29 (6)	53.0 ± 3.35 (7)	124.0 ± 7.56 (7)
60 min after water administration	42.0 ± 3.80 (6)	51.4 ± 3.39 (5)	115.5 ± 12.10 (5)	37.7 ± 3.26 (6)	55.3 ± 2.80 (6)	127.5 ± 7.78 (6)
75 min after water administration	38.4 ± 2.33 (6)	55.4 ± 3.09 (6)	126.1 ± 10.30 (6)	37.3 ± 2.20 (6)	55.5 ± 2.14 (6)	131.6 ± 4.74 (6)
90 min after water administration	40.5 ± 2.76 (6)	49.2 ± 3.05 (6)	116.6 ± 12.46 (6)	38.0 ± 2.81 (6)	51.4 ± 3.81 (6)	146.5 ± 5.18 (6)
120 min after water administration	34.8 ± 1.92 (6)	35.0 ± 2.14 (6)	127.0 ± 9.74 (6)	40.3 ± 3.55 (5)	53.7 ± 1.60 (6)	130.9 ± 9.17 (6)

Table 3 *The effect of water administration on the concentration of electrolytes of plasma and tissues in hypoproteinaemic rats*

(The values are means and standard errors Number of rats in parentheses)

Stage of observation	Cl ⁻ (m equiv /l water)	Na (m equiv /l water)	K (m equiv /l water)	Cl ⁻ (m equiv /l water)	Na (m equiv /l water)	K (m equiv /l water)
	Plasma			Muscle		
Before water administration	85.9 ± 2.77 (29)	108.3 ± 2.12 (11)	7.1 ± 0.58 (11)	46.7 ± 2.44 (29)	69.5 ± 4.22 (11)	96.0 ± 3.84 (11)
15 min. after water administration	75.6 ± 2.45 (10)	100.0 ± 1.62 (10)	6.8 ± 0.38 (10)	41.7 ± 1.87 (10)	59.5 ± 1.51 (10)	105.5 ± 3.89 (10)
30 min. after water administration	63.4 ± 1.03 (12)	97.1 ± 2.20 (8)	7.1 ± 0.82 (8)	37.6 ± 2.68 (12)	61.5 ± 5.92 (8)	93.7 ± 5.99 (8)
45 min. after water administration	71.9 ± 3.08 (16)	104.2 ± 3.40 (6)	5.8 ± 0.21 (6)	42.1 ± 1.76 (16)	61.4 ± 6.30 (6)	109.0 ± 8.27 (6)
60 min. after water administration	79.2 ± 3.20 (20)	110.8 ± 3.55 (8)	6.4 ± 0.53 (8)	51.7 ± 1.82 (20)	70.8 ± 5.00 (8)	97.4 ± 3.20 (8)
90 min. after water administration	86.8 ± 2.90 (7)	113.0 ± 3.42 (7)	6.4 ± 0.12 (7)	47.9 ± 2.68 (7)	64.7 ± 2.23 (7)	100.5 ± 10.81 (7)
	Liver			Brain		
Before water administration	36.4 ± 2.55 (11)	48.7 ± 3.24 (11)	93.8 ± 3.61 (11)	39.1 ± 3.27 (11)	59.5 ± 3.25 (11)	114.4 ± 4.44 (11)
15 min. after water administration	39.5 ± 1.11 (10)	52.3 ± 2.64 (10)	91.8 ± 0.22 (10)	39.5 ± 1.87 (10)	60.9 ± 2.02 (10)	118.5 ± 2.90 (10)
30 min. after water administration	34.6 ± 3.04 (6)	49.1 ± 3.40 (6)	89.7 ± 5.35 (6)	35.7 ± 2.77 (8)	59.1 ± 4.83 (8)	118.8 ± 10.58 (8)
45 min. after water administration	38.6 ± 4.96 (6)	46.6 ± 2.70 (8)	92.5 ± 2.48 (6)	35.1 ± 1.34 (6)	58.6 ± 1.96 (6)	110.5 ± 6.82 (6)
60 min. after water administration	39.3 ± 3.61 (8)	58.5 ± 5.77 (8)	98.5 ± 7.76 (8)	39.5 ± 2.99 (8)	60.9 ± 5.38 (8)	112.6 ± 6.23 (8)
90 min. after water administration	39.0 ± 0.97 (7)	56.1 ± 1.43 (7)	102.2 ± 13.11 (7)	40.0 ± 1.07 (7)	62.8 ± 3.24 (7)	116.0 ± 5.60 (7)

The plasma chloride concentrations were normal again in normal rats 45 min. after water administration, and in hypoproteinaemic rats 60 min. after water administration, and then remained at this level for the remaining period of observation. Chloride concentration in muscle increased over the mean values of control rats, 45 min. after water administration in normal rats ($t=3.010$, $P<0.01$) and 60 min. after water administration in hypoproteinaemic rats ($t=4.031$, $P<0.001$). The increase in the chloride concentration of muscle in the two series corresponded to the onset of water diuresis.

It is thus clear that in normal as well as in hypoproteinaemic rats a water diuresis can be divided into two stages: (a) a pre-diuretic or absorptive period, during which there was a fall of the chloride concentration in both plasma and muscle, independent of changes in their hydration; (b) an excretory period with increased water load in plasma and tissues during which the chloride concentration returned, nevertheless, to normal values in plasma and reached higher than normal values in the muscle.

Changes in the extracellular-fluid phase of tissues

The changes in the water content of plasma and the tissues, associated with changes in the concentration of electrolytes, led to variations in the amount of extracellular fluid of tissues.

It would seem from Tables 4 and 5 that the partition of water between the extracellular- and intracellular fluid phase during a water diuresis differs in normal and hypoproteinaemic animals.

In normal rats three stages could be recognized (Fig. 2): an initial decrease of the extracellular fluid phase of muscle (up to 30 min. after water administration) followed by a significant increase (45–75 min.) ending in a slow return to normal values (90–120 min.). When the changes of the extracellular fluid phase are correlated with the rate of renal water excretion, it can be seen that the initial decrease of extracellular fluid phase corresponded to the pre-diuretic period and the increase to the peak of the diuresis.

In the hypoproteinaemic rats, although the average changes in extracellular fluid phase of

Table 4 *The effect of water administration on the extracellular- and intracellular-fluid phases (chloride and sodium) of tissues in normal rats*

(The values are means and standard errors Number of rats in parentheses)

Stage of observation	Extracellular fluid phase (ml./100 g)		Muscle	Intracellular fluid phase (ml/100 g)	
	Cl ⁻	Na		Cl ⁻	Na
Before water administration	16.8±0.50 (27)	16.9±0.53 (17)		59.0±0.50 (27)	58.8±1.11 (17)
15 min. after water administration	15.3±0.27 (20)	15.4±0.47 (9)		61.5±0.40 (20)	61.5±1.23 (9)
30 min. after water administration	18.9±0.90 (18)	18.0±1.00 (11)		58.2±0.85 (18)	59.0±1.04 (11)
45 min. after water administration	20.8±0.85 (18)	21.1±0.86 (13)		56.5±0.89 (18)	56.3±0.88 (13)
60 min. after water administration	20.5±0.88 (16)	20.5±1.18 (12)		57.3±0.93 (16)	57.3±1.13 (12)
75 min. after water administration	20.6±0.66 (16)	20.7±0.70 (16)		56.7±0.63 (16)	56.7±0.64 (16)
90 min. after water administration	18.3±0.80 (12)	20.4±0.78 (12)		58.5±0.78 (12)	56.5±0.82 (12)
120 min. after water administration	17.1±0.49 (12)	16.5±0.74 (12)		59.3±0.59 (12)	59.7±0.80 (12)
Liver					
Before water administration	22.6±2.50 (11)	21.3±1.55 (10)		48.8±2.05 (11)	50.1±2.03 (10)
15 min. after water administration	25.9±2.76 (9)	23.8±2.42 (9)		45.6±1.56 (9)	47.6±1.20 (9)
30 min. after water administration	34.4±3.60 (7)	32.6±3.57 (7)		37.6±3.05 (7)	40.6±3.63 (7)
45 min. after water administration	32.0±1.40 (7)	33.4±3.90 (7)		40.8±1.43 (7)	39.2±3.80 (7)
60 min. after water administration	27.6±2.91 (6)	26.3±4.32 (5)		44.7±3.40 (6)	46.0±4.93 (5)
75 min. after water administration	30.1±2.39 (6)	30.2±3.15 (6)		41.6±1.76 (6)	41.3±3.03 (6)
90 min. after water administration	30.6±1.87 (6)	32.9±2.50 (6)		41.6±3.40 (6)	39.4±2.53 (6)
120 min. after water administration	31.5±1.70 (6)	28.1±1.95 (6)		40.3±1.75 (6)	43.7±2.30 (6)

Table 5 *The effect of water administration on the extracellular- and intracellular fluid phases (chloride and sodium) of tissues in hypoproteinaemic rats*

(The values are means and standard errors Number of rats in parentheses)

Stage of observation	Extracellular fluid phase (ml/100 g)		Muscle	Intracellular fluid phase (ml/100 g)	
	Cl ⁻	Na		Cl ⁻	Na
Before water administration	44.9±1.90 (29)	43.5±2.08 (11)		32.3±1.64 (29)	33.8±0.70 (11)
15 min. after water administration	41.9±2.16 (10)	38.8±1.24 (10)		35.5±1.86 (10)	38.7±1.24 (10)
30 min. after water administration	44.2±3.41 (12)	42.0±3.90 (8)		32.7±3.00 (12)	35.0±3.72 (8)
45 min. after water administration	46.2±3.51 (16)	44.0±4.66 (6)		31.4±3.40 (16)	33.5±4.12 (6)
60 min. after water administration	49.1±2.42 (20)	51.5±4.67 (8)		30.0±2.40 (20)	26.9±3.67 (8)
75 min. after water administration	—	—		—	—
90 min. after water administration	41.1±2.45 (7)	42.2±2.29 (7)		37.0±2.72 (7)	36.0±2.66 (7)
120 min. after water administration	—	—		—	—
Liver					
Before water administration	35.4±2.66 (11)	32.6±1.91 (11)		39.5±2.52 (11)	42.2±2.14 (11)
15 min. after water administration	39.8±1.22 (10)	37.9±2.75 (10)		35.0±1.15 (10)	37.0±3.03 (10)
30 min. after water administration	40.8±3.14 (6)	33.4±2.38 (6)		33.8±3.12 (6)	41.2±2.62 (6)
45 min. after water administration	39.4±3.42 (6)	32.3±2.02 (6)		34.9±3.59 (6)	41.8±1.87 (6)
60 min. after water administration	40.4±3.66 (8)	37.2±3.76 (8)		34.8±3.54 (8)	38.0±3.67 (8)
75 min. after water administration	—	—		—	—
90 min. after water administration	33.6±1.07 (7)	36.0±1.64 (7)		41.8±0.83 (7)	39.4±1.37 (7)
120 min. after water administration	—	—		—	—

muscle resembled closely, both in magnitude and direction, those observed in the normal animals, such changes cannot be regarded as significant owing to the large standard error of each group (Table 5)

Changes in the intracellular-water phase of tissues

It has been shown in the present series of experiments on normal rats that during a water diuresis the total water load of muscle and its extracellular-fluid phase increased in a parallel manner. But it is not clear whether these simultaneous increases are of the same magnitude, i.e. whether the increase of

the extracellular-fluid phase can be accounted for by the increase of the extra water load of the muscle.

Comparing the mean values for extracellular fluid and the total muscle water load (Tables 1 and 4) it will be seen that, whereas the extracellular-fluid phase increased from 16.8 to 20.5 ml/100 g fat-free muscle (i.e. by 3.7 ml) in 60 min., that of total water content increased from 75.8 to 77.8 ml/100 g fat-free muscle (i.e. by 2.0 ml) in the same time. The differences in the increments represents the changes in the amount of intracellular-fluid phase (Table 4)

It is thus evident that during the second or excretory stage of water diuresis an increase in the extracellular fluid phase was associated with a decrease in the amount of intracellular fluid with a concurrent increase in the extra water load of the muscle (Fig 2). In agreement with this finding is the observation that the loss of intracellular water was accompanied by a loss of potassium (Table 2). This finding agrees with that of Gamble, Blackfan & Hamilton (1925), who showed that water diuresis may cause a marked urinary excretion of endogenous potassium.

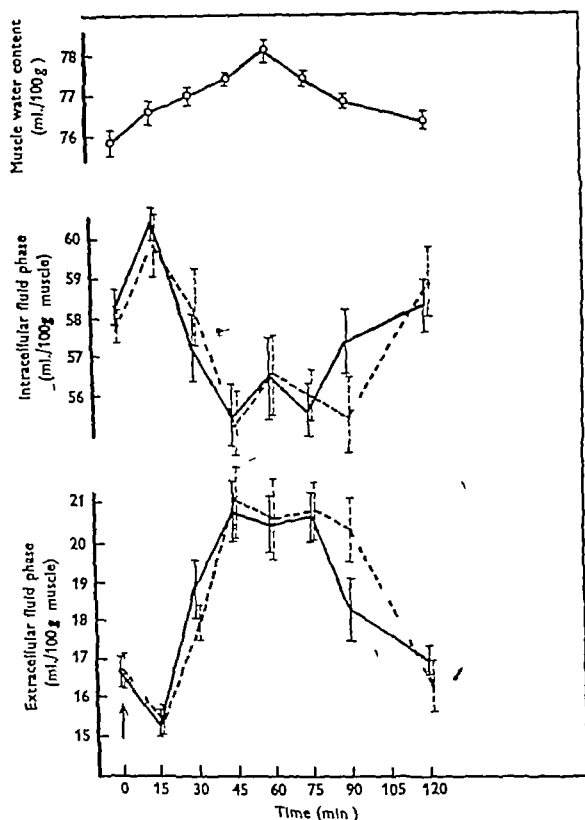


Fig 2 Changes of total water content and of the extracellular and intracellular fluid phases in the muscle of normal rats. At \uparrow 5% of body weight of water was administered. \bullet — \bullet , extracellular and intracellular fluid phases estimated in terms of chloride space, \bullet — \bullet — \bullet , extracellular and intracellular fluid phases estimated in terms of sodium space, \circ — \circ , total muscle water content. The vertical lines represent the standard error.

In hypoproteinaemic animals, on the other hand, no clear changes in the intracellular fluid phase of the tissues could be shown. The partition of water between the extracellular and the intracellular fluid phases in the tissues during a water diuresis was thus completely different in normal and hypoproteinaemic rats.

DISCUSSION

It would appear that in normal rats well defined changes in the partition of water between the extracellular- and the intracellular fluid phase of skeletal muscles corresponded to certain stages in the process of water diuresis. Following the administration of water by stomach tube, and preceding the onset of diuresis, there was a significant decrease in the extracellular-fluid phase of the muscle. When the absorptive period was reaching completion and when the renal excretion of the administered water had started, the well known increase of the muscle water load was observed. A significant increase in the extracellular fluid phase concurrent with a decrease of the intracellular fluid phase could be demonstrated at this stage. When the excretion of the extra water was nearly completed there was a return of the muscle water load and of its extracellular- and intracellular fluid phases to normal values.

These results compare with Eichelberger's (1941) findings on unanaesthetized dogs, but are in apparent contrast to those of Eggleton (1937) on anaesthetized cats. Eichelberger (1941) injected 0.9% NaCl solution intravenously into dogs, and observed that during the period of full diuresis there was a significant increase in the extracellular fluid phase of the muscle, and a slight decrease in its intracellular-fluid phase. In other words, the partition of water in Eichelberger's (1941) dogs corresponded to that observed during the excretory stage in the present series of normal rats. Eggleton (1937), on the other hand, found that the injection of 6% of the body weight of water 'into a loop of small intestine brought to the surface through a small abdominal opening' produced an increase in the total muscle water content accompanied by a decrease of its extracellular fluid phase. It should be pointed out, however, that none of Eggleton's (1937) cats was excreting the administered water, in fact, some of them had their ureters tied.

The difference between Eggleton's (1937) findings on the one hand, and those of Eichelberger (1941) and of the present series of investigations on the other, thus lies clearly in the fact that in Eggleton's (1937) cats water excretion was prevented. It is therefore likely that Eggleton's (1937) findings apply to the prediuretic or reabsorptive period only, a stage characterized by a dilution of the plasma solids and by a fall of the chloride concentration in both plasma and muscles, resulting in a decrease of the extracellular space.

In hypoproteinaemic rats the administration of the standard amount of water did not produce a significant increase of the water level in the plasma, in spite of the fact that water had been absorbed at the same rate at least as by normal rats. During the absorptive period there was a marked fall of the

plasma and muscle chloride concentrations, but in contrast to the normal animals there was no significant decrease in the extracellular-fluid phase of the skeletal muscle. Nor was there any significant increase of the extracellular-fluid phase of muscle during the post absorptive or excretory period. It would thus seem that the tissue oedema that existed initially in the hypoproteinaemic animals could not be increased to any significant extent by further administration of water.

It has indeed been suggested that in muscles in which the concentrations of chloride and sodium are increased and that of potassium decreased (i.e. in which an increase of the extracellular-fluid phase at the expense of the intracellular fluid phase obtains) the extracellular fluid phase should not be identified with an ultrafiltrate of plasma (Manery, Danielson & Hastings, 1938) but with connective tissue diluted with serum filtrate. The extracellular-fluid phase of connective tissue has been estimated to amount to almost 100% (Manery & Hastings, 1939). This interpretation may explain why an increased water load did not produce any significant increase in the extracellular-fluid phase of the muscle.

This interpretation would also provide an explanation for the delayed and diminished water diuresis displayed by hypoproteinaemic rats.

In normal rats the administration of a standard amount of water resulted in a dilution of the plasma, which according to accepted theories is indirectly responsible for the reduction of the rate of tubular water reabsorption. There is a time lag between the height of gastro intestinal water absorption and the height of the diuresis, i.e. a period during which the water absorbed is lodged in the extracellular-fluid phase of tissues, from where it ultimately returns to the blood stream to be excreted by the kidneys. In hypoproteinaemic rats the administration of a standard amount of water did not result in a further dilution of the plasma: the rate of tubular water reabsorption was thus not affected. This would explain why the water diuresis was delayed in its onset, and why the diuresis was partly regulated by an enhanced glomerular filtration rate (Dicker *et al.* 1946). As the rate at which water was absorbed from the gut was not slower than in normal rats, and as the extracellular fluid phase of skeletal muscle or liver seemed to be unable to store the amount of water absorbed, it must have been stored elsewhere. Few of the rats in this series showed any free fluid at a post mortem examination, but nearly all of them displayed perirenal and retroperitoneal connective tissues so much distended with fluid as to give them a gelatinous consistency. In several cases mediastinal connective tissue had the same appear-

ance. It must, therefore, be assumed that in hypoproteinaemic rats water administered by stomach tube is not held in the extracellular-fluid phase of muscle or liver, but that it collects in the deep connective tissue.

SUMMARY

1 A standard amount of water (5% of their body weight) was administered by stomach tube to normal and hypoproteinaemic rats, and the partition of the absorbed water between the plasma and the extracellular- and intracellular-fluid phases of certain tissues (muscle, liver and brain) was investigated.

2 In normal rats the administration of water produced effects which led to the division of the process of water diuresis into three distinct periods.

(a) The absorptive period, preceding the onset of diuresis and characterized by a dilution of the plasma accompanied by a fall in the chloride and the sodium concentration of both plasma and tissues, resulting in a decrease of the extracellular-fluid phase.

(b) The excretory period, corresponding to the height of the water diuresis accompanied by a marked increase in the chloride and sodium concentration of muscle and a decrease of potassium, resulting in an increase of the extracellular fluid phase and a decrease in the intracellular-fluid phase of the tissue, i.e. oedema of the tissue.

(c) The terminal period: return to normal values for chloride, sodium and potassium concentration in plasma and tissues.

3 In hypoproteinaemic rats water administered was absorbed at least at the same rate as in normal animals. The water absorption was finished in less than 60 min.

4 In hypoproteinaemic rats the administration of water produced a diuresis which was delayed in its onset and diminished in its volume. This abnormal response to water administration could be explained by the following findings.

(a) The administration of water did not produce any further increase in the plasma water content.

(b) No significant changes in the amount of extracellular-fluid phase per 100 g fat free muscle could be found.

(c) The water absorbed was 'visibly' collected in the perirenal and retroperitoneal connective tissue, which was so much distended as to have a gelatinous consistency.

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Chemical Constitution and Insecticidal Action

1 ORGANIC SULPHUR COMPOUNDS

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Until the discovery of the newer synthetic insecticides of the chlorinated hydrocarbon class, such as DDT and γ benzene hexachloride, organic compounds of sulphur had attracted considerable attention, and this work led to the commercial introduction of the long chain alkyl thiocyanates, to phenothiazine (originally as an insecticide but later developed as an anthelmintic) and more recently to tetraethylthiuram sulphide, an acaricide used in the treatment of scabies and mange. During the last ten years we have made an extensive investigation of the insecticidal action of organic sulphur compounds, in collaboration with the Hawthorndale Laboratory of Jealott's Hill Research Station (Imperial Chemical Industries Ltd) where the entomological testing was carried out. Although these investigations did not result in the introduction of any new commercial insecticides, nevertheless, marked insecticidal activity was found in certain types, and the correlation of structure with activity presented points of considerable interest. It is the purpose of this communication to record some of our observations on this subject.

METHODS

All compounds were submitted to routine sorting tests, and from the results the more promising were selected for extended evaluation. The extended tests necessitated the preparation of a variety of types of emulsions and dispersions (Collie, Ellingworth & Robertson, 1939, Collie,

Davies & Sexton, 1939, Harland & Sexton, 1943) suitable for the practical application of the poisons. This aspect of the research is mentioned only so far as it provides data confirming the higher activities found by the sorting tests and illustrates the selective poisoning effect on insect species.

Contact insecticides The sorting tests were carried out in the Tattersfield spraying apparatus using aphids (*Macrosiphum* spp. except where otherwise stated, the choice of species being governed by its availability at the time of the test), or adult blowflies (*Calliphora erythrocephala* Meig.) as the test insects. The compound to be tested was dissolved in ethanol, and poured into a 0.1% aqueous solution of a surface active agent of the sulphonated hydrocarbon type, the concentrations being such that the final spray contained the substance dispersed in 50% ethanol. In certain instances it was necessary to use acetone instead of ethanol. As it was appreciated that the organic solvent might play a significant part in the apparent toxicities, controls were carried out with sprays of similar composition from which the poison had been omitted, the 'absolute mortality' due to the substance under test was calculated by the formula

Percentage absolute mortality

$$= \frac{(\text{number killed by poison spray}) - (\text{number killed by control spray})}{(\text{total number of insects}) - (\text{number killed by control spray})} \times 100$$

In spite of this adjustment, the ethanol may have played a part in altering penetration of the insect cuticle by the poison (Hurst, 1943) so that the figures of 'absolute mortality' obtained may have been, considered as absolute values, false. It is felt, however, that for closely related

Table 1 *Insecticidal activities of 2-alkylthiobenzthiazoles*

Alkyl group (<i>R</i> , formula I)	Percentage absolute mortality		
	Blowflies at 0.5%	Aphids (<i>Macrosiphum</i> spp.) at 0.2%	<i>Sitophilus granarius</i> (biscuit method)
H	13*	—	0
CH ₃	100	86	99.4
C ₂ H ₅	100*	21	100
C ₃ H ₇ †	100	30	23.3
C ₄ H ₉	96	17	2.7
C ₆ H ₁₃	41	3	0
C ₇ H ₁₅	37	0	7
C ₈ H ₁₇	—	9	0
C ₈ H ₁₇ (sec)	—	17‡	0
C ₁₂ H ₂₅	4	45	0
C ₁₆ H ₃₃	0*	17	0
(CH ₃) ₂ CH	100*	24	50
(CH ₃) ₂ CHCH ₂	92*	86	7
C ₅ H ₁₁ (iso)	—	17	—
CH ₃ CHCH ₂	99*	52	13.6
C ₆ H ₅ CH ₂	62*	6	10.5
HOC ₂ H ₄	18*	—	83§
C ₂ H ₅ OC ₂ H ₄	86*	30‡	—
Standards Rotenone	100*	100	—
Lead arsenate	—	—	58.1
C ₁₂ H ₂₅ SCN	100	100	—

* Sprays contained 25% acetone instead of 50% ethanol.

† C₃H₇, C₄H₉, C₅H₁₁, etc., indicate primary straight-chain alkyl radicals unless otherwise stated

‡ Against *Doralis ruminis* L (= *Aphis ruminis* L (*fabae* Scop)) in place of *Macrosiphum* spp

§ Lead arsenate in this experiment gave 88.7% absolute mortality

groups of compounds such as those examined the 'absolute mortality' figures give a reasonably adequate comparison of the toxicities of individual members of such groups. That the toxicities were genuine was later shown by the examination of special preparations of the more active compounds.

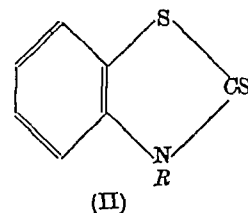
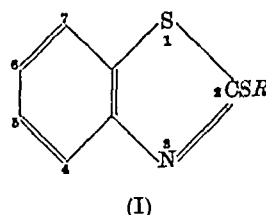
Stomach poisons. The sorting tests were carried out against *Sitophilus granarius* L (grain weevils) by a technique known as the 'biscuit' method. One part of the poison was mixed with 50 parts of flour and the whole made into a dough with 30 parts of water, the dough was dried in thin layers and broken up to provide 'biscuits' on which the insects were allowed to feed. Lead arsenate, similarly compounded, was used as a standard of comparison.

RESULTS

Tests on *S* alkylated derivatives of 2-thiobenzthiazole and 2-thiolquinoline

The contact insecticidal activity of alkyl thiocyanates (Bousquet, Salzberg & Dietz, 1935) prompted the exploration of other compounds containing the sequence of atoms —S—C—N—, and derivatives of types (I) (Colhe, Ellingworth & Robertson, 1939) and (II) (*R*=alkyl) were therefore investigated. Preparative methods for type (II) have been described elsewhere (Sexton, 1939; Reed, Robertson & Sexton, 1939). (The numbering of the benzthiazole ring is indicated in formula (I).) The results of the preliminary tests with the *S* alkylated derivatives (I) are given in Tables 1 and 2. Against blowflies, toxicity is at a maximum at C₃ in the

normal series for aphids, the methyl and isobutyl compounds were the most toxic, the normal C₆ to C₈ compounds being practically inactive. There was,



however, appreciable activity with the isooctyl and dodecyl derivatives, but the hexadecyl was again inactive. For the grain weevil there was a marked fall in toxicity after the methyl, ethyl and β-hydroxyethyl compounds.

Table 2 *Effect of concentration on toxicity to blowflies of aqueous acetone dispersions of homologous 2-alkylthiobenzthiazoles*

Alkyl group (<i>R</i> , formula I)	Percentage absolute mortality Concentration (%)				
	0.5	0.3	0.15	0.075	0.05
CH ₃	100	98	34	10	—
C ₂ H ₅	100	94	49	16	—
C ₃ H ₇	100	99	85	10	—
(CH ₃) ₂ CH	—	—	18	—	0
(CH ₃) ₂ CHCH ₂	—	—	13	—	0
CH ₃ CHCH ₂	—	—	40	—	23
Rotenone	—	—	—	—	100

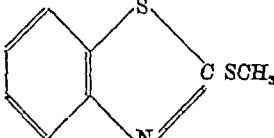
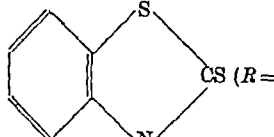
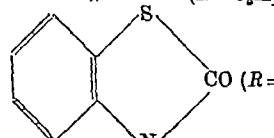
2 Ethylthiobenzthiazole, because of its toxicity to blowflies, was subjected to further evaluation. Its insecticidal effect was confirmed by the following findings in separate experiments (a) a 40 % solution in odourless kerosene gave a 68 % kill when sprayed on houseflies (*Musca domestica* L.), (b) emulsified preparations gave kills of 64–98 % at the same strength, (c) a 2 % solution in kerosene gave a 81 % kill of mosquito larvae, and (d) there was moderate toxicity to bedbugs (*Cimex lectularius* L.)

The effect of substitution in the benzene ring was examined in the cases of the 2 alkylthio derivatives (C_1 to C_4) of 6 amino, 6 nitro and 5 chloro benzthiazoles. The results will not be given in detail, but the general effect was a reduction in insecticidal activity, which was least marked in the case of the chloro compounds, against blowflies

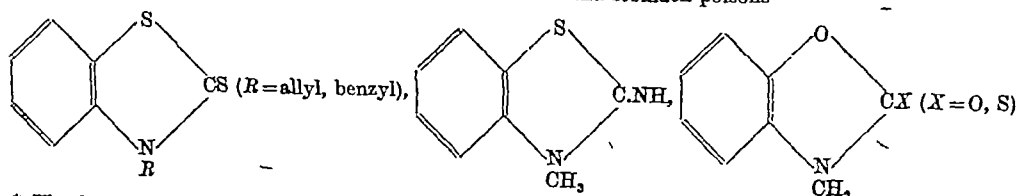
Tests with S-alkylated derivatives of 2 thiolquinoline and of 2 thiolbenzoxazole

The chemical similarity between thiazole and the corresponding pyridine derivatives suggested that this might also extend to their biological action. Accordingly several quinoline derivatives, analogous to the benzthiazole derivatives already examined, were tested against the standard insects. The results, which are not given in detail, showed that the general order of toxicity was lower than for the isosteric thiazole compounds. A notable feature was that, whereas alkylation of the thiol group in the 2 thiolbenzthiazole series raised the toxicity to the grain weevil, a reduction occurred with the quinoline compounds. The results with the other isosteric series, the benzoxazoles, showed, among the limited number

Table 3 *Insecticidal activity of alkylated benzthiazole derivatives*

Compound	Percentage absolute mortality						<i>Sitophilus granarius</i> *
	Blowflies		<i>Dorsalis ruficollis</i>		<i>Dorsalis poma</i>		
	(0.5%)	(0.2%)	(0.2%)	(0.1%)	(0.2%)	(0.1%)	
	100	73	11	8	32	—	99.4 (58.1)
	0	13	73	55	100	73	11.5 (82.3)
„ (R=C ₂ H ₅)	12	—	70	—	—	—	27.1 (82.3)
„ (R=C ₃ H ₇)	7	—	0	—	—	—	16.7 (67.7)
„ (R=C ₄ H ₉)	0	—	10	—	—	—	8.3 (67.7)
	25	—	20	—	—	—	90.5 (82.3)
„ (R=C ₂ H ₅)	12	—	70	—	—	—	—

The following had very low toxicities both as contact insecticides and stomach poisons



* The figures in brackets in this column give the percentage absolute mortality with a lead arsenate bait

of compounds examined, results parallel to those obtained with the benzthiazole derivatives in that blowflies and grain weevils were more susceptible than aphids

*Tests with N-alkylated derivatives
of 2-thiobenzthiazole*

The tests in this series were confined to the lower *N* alkylated derivatives of type (II) which were readily obtainable by methods already mentioned. An important difference in the relative susceptibility of different insects to the isomeric *S*- and *N*-alkylated pairs was revealed, for whereas the *S*-alkyl derivatives had low toxicity to aphids and high toxicity to blowflies, the *N*-methyl compound, which received the most study, was highly toxic to aphids and relatively non-toxic to blowflies. The results with these and related compounds are summarized in Table 3. In the *N*-alkyl series, the lowest member was the most active aphicide. It will be seen from Table 3 that replacement or interchange of $>S$, $>O$ and $>NH$ groups could exert marked effects, and the *S* derivative was not always the most toxic.

The aphicidal properties of the *N*-methyl compound, 2 thio 3-methylbenzthiazolone, were investigated thoroughly, and clear evidence was obtained that the activity was not due to the ethanol in the spray, indeed this may even have lowered the aphicidal activity. A concentrated emulsion was prepared by dissolving 10 parts of 2-thio 3-methylbenzthiazolone in 55 parts of cottonseed oil and emulsifying with 25 parts of water with the aid of 10 parts of a sulphonated cod oil. This emulsion, diluted with 0.1% 'Agral' Liquid Wetter, was sprayed on plants infested with several species of aphids. High kills of the insects were obtained, sometimes even at spray concentrations of less than 0.025% active ingredient. Other emulsions gave similar results. The aphicidal activity was, however, generally lower than that of dodecyl thiocyanate, and the practical utilization of this compound was limited by occasional damage to plants.

Tests on carbamates and thiocarbamates

An obvious extension of the types of compound containing adjacent S, C and N atoms was to derivatives of dithiocarbamic acid. Since, moreover, interchange of sulphur and oxygen in this class had not led to marked alterations in fungistatic activity (Davies & Sexton, 1946), or even to a more limited extent in selective herbicidal activity (Templeman & Sexton, 1946), a comparison of the insecticidal activity of carbamates and thiocarbamates was necessary. The results of contact tests are given in Table 4. As stomach poisons against *Sitophilus granarius* nearly all were less toxic than lead arsenate.

Table 4 *Contact insecticidal activity of derivatives of carbamic acid*

Compound	Percentage absolute mortality	
	Blowflies (0.5%)	Aphids (0.2%)
$C_6H_5NH \cdot COOCH_3$	58	4 (a)
$C_6H_5NH \cdot COOC_2H_5$	87	0 (a)
$C_6H_5NH \cdot CSOCH_3$	96	23 (a)
$C_6H_5NH \cdot COSCH_3$	81	0 (a)
$C_6H_5NH \cdot COSC_2H_5$	100	4 (a)
$C_6H_5NH \cdot CSOC_2H_5$	92	86 (b)
$C_6H_5NH \cdot CSOC_4H_9$	55	11 (b)
$C_6H_5NH \cdot CSSCH_3$	31	0 (a)
$C_6H_5NH \cdot CSSC_2H_5$	76	28 (a)
$C_6H_5NH \cdot CSSC_4H_9$	84	45 (a)
$C_6H_5NH \cdot CSSCH_2C_2H_5$	57	0 (a)
$C_6H_5(CH_2)_N \cdot COOCH_3$	2	0 (b)
$C_6H_5(CH_2)_N \cdot COOC_2H_5$	40	6 (b)
$C_6H_5(CH_2)_N \cdot CSSCH_3$	9	17 (b)
$C_6H_5(CH_2)_N \cdot CSSC_2H_5$	0	56 (b)
$C_6H_5(C_2H_5)_N \cdot COOCH_3$	16	23 (b)
$C_6H_5(C_2H_5)_N \cdot COOC_2H_5$	59	0 (b)
$C_6H_5(C_2H_5)_N \cdot CSSCH_3$	28	27 (b)
$C_6H_5(C_2H_5)_N \cdot CSSC_4H_9$	6	24 (b)
$(CH_3)_2N \cdot COOCH_3$	0	6 (b)
$(CH_3)_2N \cdot COOC_2H_5$	0	3 (b)
$(CH_3)_2N \cdot CSSCH_3$	—	3 (b)

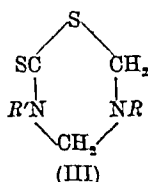
(a) *Macrosiphum* spp. (b) *Doralis rucicis* L.

Tests on thiocarbimide (isothiocyanate) derivatives

The insecticidal activity of thiocyanates differs from that of the corresponding thiocarbimides (isothiocyanates), just as does the fungistatic activity. Though dodecyl thiocyanate is a powerful contact insecticide, the corresponding isothiocyanate is much less active. Experiments with bedbugs and houseflies have shown that it is almost without action on these insects.

In the aromatic series, the thiocarbimides are powerful insecticides (Goldschmidt & Martin, 1937), and the α naphthyl compound has been developed commercially as a fly spray. The starting point for the results recorded here was phenylthiocarbimide. During a search for new compounds having a high toxicity to locusts, it was observed that this substance was highly toxic when used in a poison bait, but its volatile nature precluded its practical application. Attention was, therefore, directed to less volatile derivatives which might be expected to break down to phenylthiocarbimide *in vivo*, and the reaction products of phenyldithiocarbamic acid with formaldehyde and ammonia or primary amines, the so called 'carbothialdines', were studied in some detail. During the course of this work their constitution as reduced thiadiazines (III) was established (Ainley, Davies, Gudgeon, Harland & Sexton, 1944). The first substance examined was 'phenylmethylene carbothialdine' ($R=H$, $R'=C_6H_5$), which was obtained as an amorphous powder by treating ammonium phenyldithiocarbamate with formaldehyde under controlled conditions. The crude pro-

duct, which could not be purified because of its instability, proved highly toxic to locusts. More stable, crystalline compounds of the same type were obtained by using primary amines in place of ammonia, thus giving 'carbothialdines' in which $R = \text{alkyl}$ the preparation and properties of these compounds are described elsewhere (Amley *et al* 1944, Gudgeon, Harland & Sexton, 1943). In testing as locust poisons, the substances were ground with



an equal weight of talc, and the resulting 50 % dusts were made into baits by mixing with bran, molasses and water in the proportions 1 : 56 : 2 : 84. Locusts (*Locusta migratoria migratorioides* R & F 3rd instar hoppers) were allowed to feed on the mixtures, and the mean mortality determined after 20 hr. a similarly compounded sodium arsenite bait was used as a standard. The results with a series of compounds (Table 5) showed that substitution in the phenyl

Table 5 Toxicity of compounds of formula (III) to locusts

Constitution		Mean percentage mortality after 20 hr (sodium arsenite = 8 %)
R'	R	
C_6H_5	CH_3	100
	C_6H_5	7
	C_2H_5	100
	C_4H_9	53
	$(\text{CH}_2)_5\text{CHCH}_3$	68
	cyclohexyl	<10
	$\text{C}_6\text{H}_4\text{OH}$	100
	$-\text{CH}_2\text{CH}_2-^*$	98
	$-(\text{CH}_2)_6-^*$	<10
$(p)\text{CH}_3\text{C}_6\text{H}_4$	CH_3	88
$(p)\text{ClC}_6\text{H}_4$	CH_3	62
$(p)\text{HOC}_6\text{H}_4$	CH_3	<10
CH_3	CH_3	88
	C_2H_5	74
	$-\text{CH}_2\text{CH}_2-^*$	<10
C_2H_5	C_2H_5	74

* These are double molecules comprising two heterocyclic nuclei united by the divalent group R .

group reduced the toxicity, and that the nature of the group R also had a marked effect. The result with the second compound in Table 5 appears anomalous. Further tests, in which the amount of poison in the bait was reduced showed that no compound was more toxic than (III) ($R = \text{CH}_3$, $R' = \text{C}_6\text{H}_5$). This was next evaluated against different insects, when it was observed to be highly toxic to crickets and to the large white cabbage butterfly caterpillar (*Pieris brassicae* L.). It was of low toxicity to winter

moth larvae (*Operophtera brumata* L.), and non toxic to the diamond back moth (*Plutella maculipennis* Curt.) and to aphids. This compound, therefore, provides another outstanding example of selective insecticidal action.

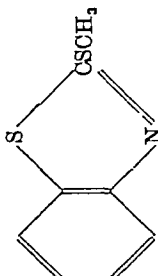
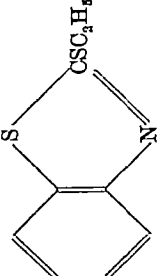
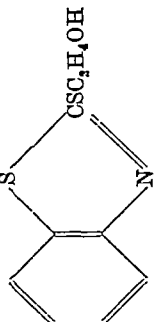
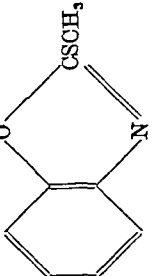
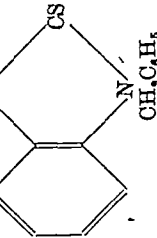
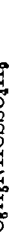
The relative susceptibility of different insect species

The previous tables show that individual compounds have considerable selective insecticidal action against aphids or blowflies. This is characteristic of organic compounds, not only when employed as contact insecticides, but also, as can be seen from the rapidly growing literature on the subject, in their antibacterial, antiprotozoal, fungicidal and herbicidal activities. It is further illustrated in the case of insecticidal action by the figures in Table 6 where stomach poison tests are considered. The first three columns refer to tests by the 'biscuit' method, and the last five columns to tests in which various insect larvae were allowed to feed on foliage sprayed with dispersions of the insecticides. In these tests the mortality by lead arsenate, which was used as a standard of comparison, was 100 % unless otherwise indicated by a figure in brackets. An outstanding example of this selective toxicity is the first compound in the list, 2-methylthiobenzthiazole, which was very toxic to *Sitophilus granarius*, *Ephestia lühmella*, vanessids and *Hypocrita jacobaeae*, but relatively non toxic to *Corcyra cephalonica* and *Operophtera brumata*.

DISCUSSION

Läuger, Martin & Muller (1944) have put forward the hypothesis regarding contact insecticidal action that the molecule should contain a grouping responsible for its toxic action, and a 'carrier' grouping conferring lipid solubility enabling the compound to pass through the fatty and lipoprotein layers of the insect cuticle. It is of interest to examine this hypothesis further, particularly in the light of the results now recorded. As Läuger *et al* (1944) point out it is difficult to decide in complicated molecules such, for example, as the pyrethrins, which part represents the toxic grouping and which the lipophilic carrier. We would prefer to speak of (a) the chemically functioning group or groups of the molecule, and (b) the physico-chemical properties, for example, of lipid and water solubility, surface activity, etc. of the molecule as a whole. By chemically functioning group is meant the group which is responsible for attachment to cell constituents. The force of attachment may not be strong in absolute terms, but if the union is of sufficient stability to disturb a delicate balance of dynamic biochemical processes then biological activity may result. This conception of the dual contribution towards biological activity should not be applied too rigidly for the two factors may, to

Table 6 Percentage absolute mortality (stomach poison tests) by several compounds against different insect larvae

Compound	Tests by 'biscuit' method			Tests on foliage			
	Flour moth (<i>Ephestia</i> <i>kühniella</i> Zell.)	Rice moth (<i>Corcyra</i> <i>cephalonica</i> Staint.)	Grain weevil (<i>Sitophilus</i> <i>granarius</i> L.)	Winter moth (<i>Operophtera</i> <i>brunata</i> L.)	Small tortoise- shell (<i>Aglais</i> <i>urticae</i> L.)	Peacock (<i>Nymphalis</i> <i>itisio</i> L.)	Diamond back moth (<i>Plutella</i> <i>maculipennis</i> Curt.)
	100 (25.1)*	26.5 (88.7)	99.4 (58.1)	13.8	100	97.5	—
	81 (25.1)	—	100 (58.1)	19.5	100	91	—
	23.1 (20.6)	—	83 (88.7)	—	—	0	7.1 (27.1)
	—	—	95.2 (13)	—	—	0	—
	—	—	0.7 (60.6)	—	—	—	9.3 (27.1)
	—	—	—	44.5	—	2.2	—

* Mortality with lead arsenate (see text)

some extent, be interdependent. The chemically functioning groups may have their contribution to make towards physical properties, particularly when the lesser interatomic forces such as those of the hydrogen bond are concerned. Physical causes may predominate in insecticidal action, and in these instances certain characteristic effects can be observed. These have been considered in relation to biological activity generally on a thermodynamic basis by Ferguson (1939), and they include the effects on variation in homologous series, including the peak effect noted in the aphicidal properties of alkyl thiocyanates, and in several instances reported in this paper. Another characteristic manifestation of physical mechanisms is the varying response of different biological species (cf Ferguson, 1939) to the same compound. Marked specificity of insecticidal action is recorded in this paper. This is probably a more general effect of organic insecticides than has been realized, for the more potent contact insecticides are usually used at such high strengths relative to their absolute potency that selective effects may be obscured. The chemically functioning groups in the compounds reported here are not identical, although the compounds nearly all contain the sequence of atoms $-S-C-N-$. In the powerfully insecticidal aliphatic thiocyanates, the critical feature of the molecule is probably the ability of the carbon atom of the $-SCN$ group to accept electrons (Hoggarth & Sexton, 1947). In the 2 alkylthiobenzthiazoles and the isomeric *N*-alkyl compounds, on the contrary, donation of electrons would appear to be the characteristic function leading to chemical reactivity, for they readily give addition compounds with bromine and with copper salts. Much more drastic experimental conditions are required for other types of reactions, for example, hydrolysis, requiring electron accession to the carbon atom. As already mentioned, the cyclic type of compound (III) is believed to act through conversion to the aryl thiocarbimide which in turn reacts with a cell constituent. Although there is no conclusive evidence for this breakdown *in vivo*, the conception is supported by the finding (hitherto un-

published) that the fungistatic activity of compounds of type (III), in which substituents in the aryl nucleus are varied, exactly parallels that of the corresponding thiocarbimides (Davies & Sexton, 1946).

It is to be noted that the interchange of oxygen and sulphur in the carbamates (Table 5) has minor effects which might well be ascribed to no more than the differences in physical properties. The same absence of major effect on substitution of oxygen for sulphur was observed in the fungistatic activities of these substances (Davies & Sexton, 1946).

SUMMARY

1 The toxicities by contact and by stomach action of organic sulphur compounds of the following classes to various insects are recorded: benzthiazole derivatives and related quinoline and oxazole compounds, thio- and dithio carbamates, substituted thiadiazines ('carbothialdines').

2 There is a marked selectivity in the action of the various substances against different insect species. Marked contact aphicidal activity is shown by 2 thio-3-methylbenzthiazolone (II, $R = CH_3$) and high toxicity to locusts (stomach poison) by the thiadiazine (III) ($R = CH_3$, $R' = C_6H_5$).

3 The results as a whole are discussed in relation to the hypothesis of Luger, Martin & Muller (1944) which relates contact insecticidal action to the presence in the molecule of a 'toxic' group and a 'carrier' group, and it is concluded that the physical properties of the molecules concerned play a dominating part in deciding their insecticidal activity.

We are indebted to Prof. A. Robertson of Liverpool University and Dr F. P. Reed for the original preparations of 2 alkylthiobenzthiazoles and the preparation of the quinoline derivatives. Certain other compounds were prepared as described in papers referred to in the text by Dr A. D. Ainley, Dr H. Gudgeon and Dr J. C. Harland. The emulsions were prepared by Dr B. Collie. We wish to make acknowledgement also to the entomologists at Hawthorne Dale Laboratories, Jealott's Hill Research Station, where the biological testing methods were devised and the routine evaluations carried out, and particularly to Messrs M. D. Price and F. J. D. Thomas who were associated with this work from its commencement.

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Chemical Constitution and Insecticidal Action

2 SUBSTITUTED α AMINONITRILES

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(Received 5 March 1948)

The high contact insecticidal activity of certain thiocyanic esters of alcohols of appropriate molecular weight and stereochemical composition prompted the investigation of other compounds containing the cyano group. So far as is known, the insecticidal activity of nitriles is markedly less than that of the corresponding thiocyanates. From our own observations, for example, lauronitrile has only a fraction of the aphicidal activity of dodecyl thiocyanate. If, as has been suggested by Hoggarth & Sexton (1947), the insecticidal activity of the thiocyanates is in part due to the special chemical reactivity of the carbon atom in the SCN group, then it follows that the introduction of other groups which modify the reactivity of the C atom of the CN group in a similar way may be expected to give compounds having insecticidal properties.

The cyanamides were considered since they, like the thiocyanates, have the CN group separated from the rest of the molecule by a heteroatom, but an investigation with Dr W H Davies showed that their high chemical reactivity (notably their tendency to polymerization) rendered them unattractive for the purpose in view. The separation of the nitrogen atom from the CN group gives the α aminonitriles, a class of compound which is readily prepared with a variety of structural permutations. Although these have additional reactive features (see Discussion, p 472) the CN group can be hydrolyzed under appropriate experimental conditions, as can the CN group in the thiocyanates. It was, therefore, decided to conduct a systematic investigation into their insecticidal activity. Some very powerful insecticides were thereby discovered from amongst the many dozens of compounds examined (see Collie, Hill & Sexton, 1939, Sexton, 1939).

Our work was interrupted by the war, and since then the appearance of the newer chlorinated hydrocarbon contact insecticides has not encouraged us to reopen the investigation. Nevertheless, points of considerable theoretical interest emerged, and this communication records such of the results as have a bearing on the problem of correlating constitution with activity. Since our work was carried out, certain compounds of this broad chemical class have been found by Siegler & Gertler (1946) to be toxic

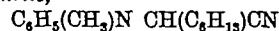
to codling moth larvae (*Cydia pomonella* L.), but so far as we are aware they have not been mentioned in any other scientific publication.

METHODS

Preparation of compounds

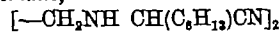
The general preparative methods are based upon the principle of condensing an aldehyde or ketone cyanohydrin with NH_3 or a primary or secondary amine, and four variations of experimental technique were employed (Jay & Curtius, 1894, Knoevenagel, 1904, Kötze & Merkel, 1926, Walther & Hübner, 1916). Trimeric methyleneaminoacetonitrile (' α hydroformamine cyanide') was obtained by the 'literature' method (*Organic Syntheses*, Coll. Vol. 1, 348, Johnson & Rinehart, 1924). Many of the other compounds had already been described in the literature, but some were new, and where these had marked biological activity, their identity was checked by elementary analysis. Details of the preparation of three of the most important of the new compounds are appended, and Table I gives the properties of all the new compounds.

α Phenylmethylaninoctoic nitrile,



Purified heptaldehyde (405 ml.) was added during 30 min. with stirring to 40% NaHSO_3 solution (615 ml.). The hot solution was then treated with methylamine (360 ml.) during 20 min., during which time the reaction mixture assumed a jelly like consistency. After 2.5 hr. at 70–75° the mass became transparent and fluid. A solution of 80% NaCN (180 g.) in water (360 ml.) was then added during 20 min., the temperature being kept below 70°. Stirring was continued for a further 20 min., and after addition of 1 l. of water, the supernatant oil was separated, washed and dried. Yield, 567 g. (82% of theory on heptaldehyde). The material had b.p. 156–157°/2 mm., and when freshly distilled was a white fluorescent oil. On storage it gradually turns yellow. (Found C, 78.85, H, 10.0, N, 11.7. $\text{C}_{15}\text{H}_{27}\text{N}_2$ requires C, 78.3, H, 9.6, N, 12.1%.)

$\alpha\beta$ Bis(α' -cyanoheptylamino)ethane,



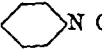

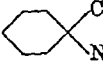
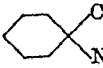
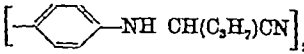
Purified heptaldehyde (134 ml.) was added to 40% NaHSO_3 solution (310 ml.). The solution was maintained at 60° for 30 min., when 90% ethylenediamine (37 ml.) was added. After 2 hr. stirring at 60°, a solution of 90% NaCN (56 g.) in water (172 ml.) was run in (30 min.), and after a further 30 min. the oil was separated and washed first with brine, then twice with water. It was dried over MgSO_4 and slowly solidified, giving white plates (from light petroleum), m.p. 45°. (Found C, 70.3, H, 10.6, N, 18.1. $\text{C}_{18}\text{H}_{34}\text{N}_4$

requires C, 70.6, H, 11.1, N, 18.3%) Treatment in glacial acetic acid with NaNO_2 gave the dinitroso derivative, m p 84–87° (Found N, 22.6 $\text{C}_{11}\text{H}_{13}\text{O}_2\text{N}_4$ requires N, 23.1%)

As originally prepared, this substance was an oil, and it was only when further preparations were made that it was eventually obtained in the solid state. Solidification of the oil is sometimes extremely slow, and the earlier tests

were performed with liquid preparations. Biological tests showed, however, that there was practically no difference in aphicidal activity between the original liquid preparations and the pure solid, and the original preparations are considered to consist substantially of the desired material. Heptaldehyde cyanohydrin, a possible contaminant in the liquid material, was not aphicidal.

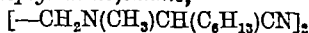
Table 1 Preparation and properties of new aminonitriles

No	Formula	Physical constants
23	$\text{C}_6\text{H}_5\text{NH CH}(\text{CH}_3)\text{CN}$	M p 90–91°
27	$\text{HOC}_2\text{H}_4\text{NH CH}(\text{C}_6\text{H}_5)\text{CN}^*$	B p 126–128°/20 mm
24	$\text{C}_6\text{H}_5(\text{CH}_2)_2\text{N CH}(\text{C}_2\text{H}_5)\text{CN}$	B p 162–164°/15 mm
20	$\text{C}_6\text{H}_5(\text{C}_2\text{H}_5)_2\text{N CH}(\text{C}_2\text{H}_5)\text{CN}$	B p 155–156°/15 mm
29	$\text{HOC}_2\text{H}_4\text{NH CH}(\text{C}_6\text{H}_5)\text{CN}$	B p 160°/16 mm
11	$(\text{CH}_3)_2\text{N CH}(\text{C}_6\text{H}_{13})\text{CN}$	B p 120°/16 mm
12	$(\text{C}_4\text{H}_9)_2\text{N CH}(\text{C}_6\text{H}_{13})\text{CN}$	B p 171°/17 mm.
16	$(\text{C}_6\text{H}_{11})_2\text{N CH}(\text{C}_6\text{H}_{13})\text{CN}$	B p 185–190°/18 mm
30	$\text{C}_6\text{H}_{11}\text{NH CH}(\text{C}_6\text{H}_{13})\text{CN}$	B p 170°/18 mm
31	$\text{cycloC}_6\text{H}_{11}\text{NH CH}(\text{C}_6\text{H}_{13})\text{CN}$	B p 150°/16 mm
15	 $\text{N CH}(\text{C}_6\text{H}_{13})\text{CN}$	B p 156°/16 mm
15a	 $\text{N CH}(\text{C}_6\text{H}_{13})\text{CN}$	Low m p, b p 166°/16 mm
28	$\text{HOC}_2\text{H}_4\text{NH CH}(\text{C}_6\text{H}_{13})\text{CN}$	M p 78–80°
26	$\text{C}_6\text{H}_5\text{NH CH}(\text{C}_6\text{H}_{13})\text{CN}$	B p 170°/17 mm
17	$\text{C}_6\text{H}_5(\text{CH}_2)_2\text{N CH}(\text{C}_6\text{H}_{13})\text{CN}$	B p 156–157°/2 mm
19	$\text{C}_6\text{H}_5(\text{C}_2\text{H}_5)_2\text{N CH}(\text{C}_6\text{H}_{13})\text{CN}$	B p 192–194°/18 mm
36	$\text{cycloC}_6\text{H}_{11}\text{NH C}(\text{CH}_3)_2\text{CN}$	M p 52–53°
37	$\text{HOC}_2\text{H}_4\text{NH C}(\text{CH}_3)_2\text{CN}$	B p 101–103°/27 mm
42	$\text{C}_6\text{H}_5\text{NH C}(\text{CH}_3)[\text{CH}_2\text{CH}(\text{CH}_3)_2]\text{CN}$	M p 52–53°
45	 $\text{NH C}_2\text{H}_4\text{OH}$	M p 72–73°
47a	 $\text{N}(\text{C}_2\text{H}_5)_2$	Liquid
10	$(\text{C}_4\text{H}_9)_2\text{N CH}(\text{C}_{17}\text{H}_{35})\text{CN}$	Waxy solid
63	$[-\text{CH}_2\text{NH C}(\text{CH}_3)(\text{C}_2\text{H}_5)\text{CN}]_2$	M p 46–47°
53	$[-\text{CH}_2\text{N}(\text{CH}_3)\text{CH}(\text{C}_6\text{H}_{13})\text{CN}]_2$	M p 48–51°
57	$\left[\begin{array}{c} -\text{CH}_2- \\ -\text{CH}_2- \end{array} \right] \text{N CH}(\text{CH}_3)\text{CN} \left. \vphantom{\begin{array}{c} -\text{CH}_2- \\ -\text{CH}_2- \end{array}} \right]_2$	M p 165°
58	$\left[\begin{array}{c} -\text{CH}_2- \\ -\text{CH}_2- \end{array} \right] \text{N CH}(\text{C}_2\text{H}_5)\text{CN} \left. \vphantom{\begin{array}{c} -\text{CH}_2- \\ -\text{CH}_2- \end{array}} \right]_2$	M p 149–151°
59	$(p)\text{C}_6\text{H}_4[\text{NH CH}(\text{C}_2\text{H}_5)\text{CN}]_2$	M p 116–118°
60	$(p)\text{C}_6\text{H}_4[\text{NH CH}(\text{C}_6\text{H}_{13})\text{CN}]_2$	M p 145°
51	$[-\text{CH}_2\text{CH}_2\text{CH}_2\text{NH CH}(\text{C}_2\text{H}_5)\text{CN}]_2$	Undistillable liquid
52	$[-\text{CH}_2\text{CH}_2\text{CH}_2\text{NH CH}(\text{C}_6\text{H}_{13})\text{CN}]_2$	Undistillable liquid
61	 $\text{NH CH}(\text{C}_2\text{H}_5)\text{CN} \left. \vphantom{\text{NH CH}(\text{C}_2\text{H}_5)\text{CN}} \right]_2$	M p 182–184°
54	$[-\text{CH}_2\text{NH C}(\text{CH}_3)(\text{C}_{11}\text{H}_{23})\text{CN}]_2$	Undistillable liquid

The new compounds listed in this table were not subjected to elementary analysis since (a) they were all of the same chemical type, prepared by standardized methods, and (b) their physical properties were in accord with expectations from the properties of near homologues, which have been fully characterized and described in the literature. For some of the more important new compounds, analyses are appended to the detailed preparative descriptions given on pp 468–470.

* C_2H_7 , C_4H_9 , C_6H_{11} , etc indicate normal primary straight-chain alkyl radicals unless otherwise stated.

$\alpha\beta$ Bis(*N*-methyl α' cyanoheptylamino)ethane,



Redistilled heptaldehyde (22.8 g) was slowly run into 40% NaHSO₃ (57.2 ml.) at 60–70°. After 30 min a solution of *NN'* dimethylethylenediamine dihydrochloride (16.1 g) in 2*N*-NaOH (100 ml) was added. After further stirring (1 hr) at the same temperature, a solution of NaCN (12 g) in water (18 ml.) was added. The oil which separated was washed with brine, and dried with MgSO₄, yield, 32.8 g (94% of theory). It could not be distilled without decomposition, but it solidified slowly and had m.p. 48–51° (from methanol) (Found C, 71.95, H, 10.8, N, 17.3. C₂₆H₃₈N₄ requires C, 71.8, H, 11.4, N, 16.8%)

Biological testing

Sorting tests were carried out using aphids (usually the chrysanthemum aphid, *Macrosiphoniella sanborni* G, but the bean aphid, *Doralis rumicis* L (= *Aphis rumicis* L, (*fabae* Scop)), when the former were not available) and adult blowflies (*Calliphora erythrocephala* Meig) with contact spraying in the Tattersfield apparatus, and for stomach poisoning effects using the grain weevil, *Sitophilus granarius* L, which was allowed to feed on 'biscuits' prepared from flour and water containing the substance under test. These methods, including the methods used for the preparation of the sprays, are described by Davies & Sexton (1948), in brief, the dispersions for spraying were made by adding ethanolic solutions of the substances under test to a dilute solution of a dispersing agent, so that the final sprays contained 50% ethanol. The possible interfering effect of the ethanol was appreciated, and the activity of promising substances was confirmed by the examination of specially prepared dispersions. The ethanol dispersion technique served to provide a standard method of presentation, applicable to most of the compounds examined, and so to permit comparisons of activity in groups of closely related compounds. Cases where this dispersion technique was not applicable are noted in the tables. In the stomach poison tests, lead arsenate was used as a standard of comparison, and the figures in brackets in Table 2 are the toxicities of lead arsenate in the individual experiments. Toxicities are expressed as 'absolute mortality', a figure derived after adjustment for mortalities caused by sprays or 'biscuits' from which the poison was omitted (Davies & Sexton, 1948).

RESULTS

The earliest tests were stomach poison tests, and, using the 'biscuit' method, α -hydroformamine cyanide was found to be highly toxic not only to the grain weevil, but also to the flour moth, *Ephestia kuehniella* Zell, and moderately toxic to leather-jackets. It was without effect on rice moth larvae (*Corcyra cephalonica* Staint.) Immodiacetonitrile, NH(CH₂CN)₂, was also toxic to the grain weevil, but the methylene derivative of this, CH₂[N(CH₂CN)]₂, was non toxic. It was these results which led to the decision to conduct a systematic investigation into the α -aminonitriles. The results of the sorting tests are summarized in Table 2.

The results of the above preliminary evaluation showed that high aphicidal activity could be

obtained with compounds of the aminonitrile class. The next stage of the investigation was, therefore, a further examination of the more promising compounds at greater dilutions, and also an extension of the investigation to compounds derived from diamines. In selecting compounds for further study some account was taken of their potential availability. The results of the further sorting tests, using the standard ethanol dispersion method on chrysanthemum aphid in the Tattersfield apparatus, are given in Table 3.







Since most of the figures for mortality in Table 3 had been obtained by using the 50% ethanol sprays, it was necessary to make compositions which could be diluted with water to provide sprays which were essentially aqueous in nature, though the presence of a surface active agent as emulsifier was of course necessary. For this purpose concentrated emulsions of the following composition by weight were prepared and were diluted either with water or with 0.1% 'Agral 2' (wetting agent) and sprayed on chrysanthemum aphid using the Tattersfield apparatus.

- 1 C₆H₅(CH₂)N CH(C₆H₁₃)CN (Compound 17) 54%, ethanolamine oleate 36%, ethoxyethanol 10%.
- 2 [-CH₂NH CH(C₆H₁₃)CN]₂ (Compound 49) 15.6%, sulphonated cod oil 38.3%, ethoxyethanol 46.1%.
- 3 Compound 49, 20%, 'Agral' Liquid Wetter 43%, ethoxyethanol 37%.
4. Compound 49, 8.3%, castor oil 16.7%, trichloroethylene 47%, casein 4.5%, borax 2.1%, water 64.5%.
- 5 Compound 49, 9.1%, triethanolamine oleate 89.1%, ethoxyethanol 1%.
- 6 [-CH₂NH CH(C₆H₁₃)CN]₂ (Compound 50) 20%, 'Agral' Liquid Wetter 43%, ethoxyethanol 37%.
- 7 Compound 50, 15.6%, sulphonated cod oil 38.3%, ethoxyethanol 46.1%.
- 8 Compound 50, 10%, castor oil 10%, trichloroethylene 45%, casein 4.3%, borax 2.1%, water 64.5%.

The results of spraying tests with the above emulsions at 0.05% active ingredient are given in Table 4. Clearly, although the degree of activity was considerably affected by the nature of the emulsion, the high aphicidal activity revealed by the sorting test was genuine. This was further confirmed by trials of dry dust preparations (3% on talc) against bean and hop aphid, *Phorodon pruni* Scop, on infested plants, with nicotine and 2-thio-3-methylbenzthiazolone (Davies & Sexton, 1948) included for comparison. The results are given in Table 5. Possibly the superiority of nicotine in this experiment was due to its fumigant action. It may be mentioned at this point that, in experiments on infested plants, marked variations in phytocidal activity between the different emulsions were also noted, and that the most effective emulsions from the aphicidal point of view were not necessarily the most phytocidal.

Table 2 *Insecticidal sorting tests with (mono) α -aminonitriles*

TABLE 2. Toxicity of various compounds

No	Compound	Conc (%)	Percentage absolute mortality				Grain weevils (‘biscuit’ method)	
			Aphids		Blowflies			
			0.35	0.2	0.55	0.35		
1	$\text{N}(\text{CH}_2\text{CN})_2$		28	—	—	17	—	
2	$(\text{CH}_3)_2\text{NCH}_2\text{CN}$		—	3 (b)	50	—	22 (58)*	
3	$(\text{C}_2\text{H}_5)_2\text{NCH}_2\text{CN}$		—	6 (b)	69	—	8 (58)	
4	$(\text{C}_4\text{H}_9)_2\text{NCH}_2\text{CN}$		—	3 (b)	97	—	57 (58)	
5	 NCH ₂ CN		—	3 (b)	85	—	10 (58)	
6	$\text{C}_6\text{H}_5(\text{CH}_2)_2\text{NCH}_2\text{CN}$		—	3 (b)	27	—	83 (47)	
7	$(\text{C}_6\text{H}_5)_2\text{NCH}(\text{CH}_3)\text{CN}$		—	28 (b)	17	—	8 (92)	
8	$(\text{C}_2\text{H}_5)_2\text{NCH}(\text{C}_6\text{H}_{13})\text{CN}$		—	88 (b)	99	—	88 (44)	
9	$(\text{C}_6\text{H}_5)_2\text{NCH}(\text{C}_6\text{H}_5)\text{CN}$		—	34 (b)	100	—	99 (44)	
10	$(\text{C}_2\text{H}_5)_2\text{NCH}(\text{C}_{17}\text{H}_{35})\text{CN}$		97	18†	—	4	—	
11	$(\text{CH}_3)_2\text{NCH}(\text{C}_6\text{H}_{13})\text{CN}$		77	—	92	—	78 (50)	
12	$(\text{C}_4\text{H}_9)_2\text{NCH}(\text{C}_6\text{H}_{13})\text{CN}$		100	—	100	—	16 (50)	
13	$(\text{C}_4\text{H}_9)_2\text{NCH}(\text{CHCH}_2\text{CN})\text{CN}$		100	—	—	18	—	
14	$(\text{HOC}_2\text{H}_4)_2\text{NCH}(\text{C}_6\text{H}_{13})\text{CN}$		41, 25†	—	—	3, 0†	—	
15	 NCH(C ₆ H ₁₃)CN		100	—	100	—	54 (50)	
15a	 NCH(C ₆ H ₁₃)CN		90†	—	58†	—	—	
16	$(\text{C}_6\text{H}_{11})_2\text{NCH}(\text{C}_6\text{H}_{13})\text{CN}$		100†	—	—	19†	—	
17	$\text{C}_6\text{H}_5(\text{CH}_2)_2\text{NCH}(\text{C}_6\text{H}_{13})\text{CN}$		100	—	—	19	3 (50)	
18	<i>cyclo</i> $\text{C}_6\text{H}_{11}(\text{C}_2\text{H}_5)_2\text{NCH}(\text{C}_6\text{H}_{13})\text{CN}$		100	—	—	—	20 (95)	
19	$\text{C}_6\text{H}_5(\text{C}_2\text{H}_5)_2\text{NCH}(\text{C}_6\text{H}_{13})\text{CN}$		28†	—	—	58†	—	
20	$\text{C}_6\text{H}_5(\text{C}_2\text{H}_5)_2\text{NCH}(\text{C}_3\text{H}_7)\text{CN}$		53, 4†	—	—	96, 81†	56 (95)	
22	$\text{H}(\text{C}_6\text{H}_5)_2\text{NCH}_2\text{CN}$		—	17 (b)	31	—	90 (45)	
23	$\text{H}(\text{C}_6\text{H}_5)_2\text{NCH}(\text{CH}_3)\text{CN}$		37	—	—	4	21 (42)	
24	$\text{C}_6\text{H}_5(\text{CH}_2)_2\text{NCH}(\text{C}_6\text{H}_7)\text{CN}$		64†	—	—	54†	12 (41)	
25	$\text{H}(\text{C}_6\text{H}_5)_2\text{NCH}(\text{C}_3\text{H}_7)\text{CN}$		27	—	—	37	10 (41)	
26	$\text{H}(\text{C}_6\text{H}_5)_2\text{NCH}(\text{C}_6\text{H}_{13})\text{CN}$		54	—	59	—	8 (50)	
27	$\text{HOC}_2\text{H}_4\text{NHCH}(\text{C}_3\text{H}_7)\text{CN}$		56	—	—	9	9 (95)	
28	$\text{HOC}_2\text{H}_4\text{NHCH}(\text{C}_6\text{H}_{13})\text{CN}$		100	100	22	—	2 (59)	
29	$\text{HOC}_2\text{H}_4\text{NHCH}(\text{C}_6\text{H}_5)\text{CN}$		39	—	16	—	11 (59)	
31	<i>cyclo</i> $\text{C}_6\text{H}_{11}\text{NHCH}(\text{C}_6\text{H}_{13})\text{CN}$		100	—	99	—	20 (56)	
35	$\text{H}(\text{C}_6\text{H}_5)_2\text{N}(\text{C}(\text{CH}_3)_2)\text{CN}$		54	—	—	48	8 (41)	
40	$\text{H}(\text{C}_6\text{H}_5)_2\text{N}(\text{C}(\text{CH}_3)(\text{C}_2\text{H}_5))\text{CN}$		86	71†	—	65	5 (41)	
42	$\text{H}(\text{C}_6\text{H}_5)_2\text{N}(\text{C}(\text{CH}_3)(\text{C}_4\text{H}_9\beta))\text{CN}$		97	39†	—	28	6 (41)	
44	 CN		100	60†	—	10	5 (51)	
45	 CN		0	—	—	26	0 (95)	
46	 CN		82	20†	—	11	0 (42)	

* The figures in brackets in this column give the mortality with a lead arsenate bait

† Emulsified by means of a sulphonated oil.

The chrysanthemum aphid was used except where (b) indicates the bean aphid

In addition to the compounds listed above the following gave absolute mortalities of not more than 15 % to all three insects


No	Compound	No	Compound
21	$\text{CH}_2\text{NH CH}_2\text{CN}$	37	$\text{HOC}_2\text{H}_4\text{NH C}(\text{CH}_3)_2\text{CN}$
30	$\text{C}_6\text{H}_{11}\text{NH CH}(\text{C}_6\text{H}_{13})\text{CN}$	38	$\text{NH}_2\text{C}(\text{CH}_3)(\text{C}_2\text{H}_5)\text{CN}$
32	Benzoyl derivative of 31	39	Oxalate and tartrate of 38
33	$\text{NH}_2\text{C}(\text{CH}_3)_2\text{CN}$	43	$\text{HOC}_2\text{H}_4\text{NH C}(\text{CH}_3)(\text{C}_4\text{H}_9\beta)\text{CN}$
34	Four acyl derivatives of 33	47	
36	<i>cyclo</i> $\text{C}_6\text{H}_{11}\text{NH C}(\text{CH}_3)_2\text{CN}$	41	$\text{NH}_2\text{C}(\text{CH}_3)(\text{C}_4\text{H}_9\beta)\text{CN}$

Table 3 *Further aphicidal sorting tests with aminonitriles*

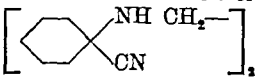
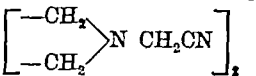
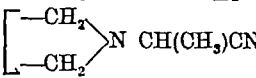
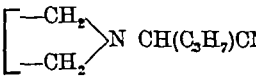
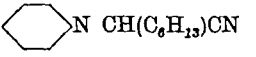
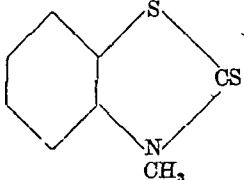
No	Compound	Conc (%)	Percentage absolute mortality of chrysanthemum aphid			
			0.35	0.2	0.05	0.025
48	$[-CH_2NH CH(CH_3)CN]_2$	100	100	98	40	—
49	$[-CH_2NH CH(C_2H_5)CN]_2$	100	100	100	96	23
50	$[-CH_2NH CH(C_6H_{13})CN]_2$	100	100	100	100	98
17	$C_6H_5(CH_3)N CH(C_6H_{13})CN$	100	100	80	35	—
51	$[-CH_2CH_2CH_2NH CH(C_2H_5)CN]_2$	100	100	100	34	—
52	$[-CH_2CH_2CH_2NH CH(C_6H_{13})CN]_2$	100	100	100	89	—
53	$[-CH_2N(CH_3) CH(C_6H_{13})CN]_2$	100	100	100	98	90
54	$[-CH_2NH CH(CH_3)(C_{11}H_{23})CN]_2$	90	90	26	16	—
55		21	21	—	—	—
56		0	0	—	—	—
57		100	100	—	—	—
58		0	0	—	—	—
59	$(p)C_6H_4[NH CH(C_2H_5)CN]_2$	8	8	—	—	—
60	$(p)C_6H_4[NH CH(C_6H_{13})CN]_2$	11	11	—	—	—
61	$[-(1-4-cyclo)C_6H_9NH CH(C_2H_5)CN]_2$	18	18	—	—	—
62	$[-CH_2NH C(CH_3)_2CN]_2$	26	26	—	—	—
63	$[-CH_2NH C(CH_3)(C_2H_5)CN]_2$	48	48	—	—	—
64	$[-CH_2NH CH(C_6H_5)CN]_2$	8	8	—	—	—
12	$(C_4H_9)_2N CH(C_6H_{13})CN$	100	100	95	58	—
15		—	—	100	77	43
31	$cycloC_6H_{11}NH CH(C_6H_{13})CN$	—	—	100	84	13
28	$HOC_2H_4NH CH(C_6H_{13})CN$	—	—	100	58	—
13	$(C_4H_9)_2N CH(CH_2CH_2CH_3)CN$	—	—	100	25	—
18	$cycloC_6H_{11}(C_2H_5)NCH(C_6H_{13})CN$	—	—	100	72	—
—	$C_{12}H_{25}SCN$	100	100	100	100	63

Table 4 *Toxicity of aminonitrile emulsions to chrysanthemum aphid with a concentration of 0.05% insecticide*

Emulsion	Percentage absolute mortality	
	Diluted with water	Diluted with 0.1% 'Agrisal 2'
1	100	—
Spray of active ingredient in 50% ethanol	49	—
2	37.5	—
3	25	—
4	69	44
5	58	47
Spray of active ingredient in 50% ethanol	62	—
6	96	98
7	98	60
8	100	100
Spray of active ingredient in 50% ethanol	100	—

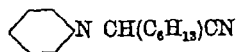
Table 5 *Toxicity of 3% dusts to aphid*

Insecticide	Percentage kill	
	Bean aphid	Hop aphid
$[-CH_2NH CH(C_6H_{13})CN]_2$	39	22
$C_6H_5(CH_3)N CH(C_6H_{13})CN$	63	47
	48	2
Nicotine	98	97.5

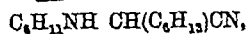
DISCUSSION

The first point which emerges from a study of the above results is the variation in toxicity to different insect species, an effect which was observed in

studies of organic sulphur compounds and which, indeed, is probably a general feature of organic insecticides (Davies & Sexton, 1948). Certain of the more potent insecticides (Table 2) showed high toxicity to all three test insects, but the toxicity was specific where evaluation at lower dosages was carried out. For example, the two compounds



and

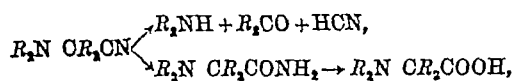


when tested at 0.2%, gave 100% absolute mortality of aphids but were non-toxic to blowflies. Again, extended evaluation of compounds having marked stomach-poison activity against the grain weevil showed that they were much inferior to lead arsenate against various leaf-eating larvae. This was true, for example, with $(\text{C}_6\text{H}_5)_2\text{N} \text{ CH}(\text{C}_6\text{H}_5)_2\text{CN}$ (Table 2) which, though more toxic than lead arsenate to the grain weevil, was practically without action against a wide range of leaf-eating larvae. The specificity of α hydroformamine cyanide as a stomach poison has already been referred to. We found phenylglycine nitrile to be more toxic than lead arsenate to the grain weevil. Siegler & Gertler (1946) found it to be less toxic than lead arsenate to the codling moth, but they found the *p*-tolyl analogue to be more toxic than lead arsenate to the codling moth.

Considering the contact insecticidal action and particularly the effects against aphids, it is clear that the physical properties of the molecule play an important part in influencing its toxicity. Consideration of Tables 2 and 3 shows numerous instances of this, including the effects of ascending an homologous series (cf. the results of Bousquet, Salzberg & Dietz, 1935, with thiocyanates). A further illustration of the importance of physical properties is provided by the effect of introducing an aliphatic hydroxyl group. This will tend to decrease the lipid/water partition, a property which is of profound importance in certain narcotics, and which may be equally relevant in contact insecticides. If this is so, it is to be expected that the introduction of a hydroxyl group may produce changes as great as the move of several steps in an homologous series. A good instance of this is seen in the comparison of compounds 8 and 14 (Table 2). Of the compounds containing aliphatic OH, only one (no. 28, Tables 2 and 3) showed marked aphicidal activity. Clearly, therefore, the inclusion of such groups is not necessarily deleterious, but until more is known of the precise relationships of water and lipid solubility with insecticidal activity, no predictions are possible. Another physicochemical factor of apparent significance is that of molecular shape. This is illustrated by the comparison of two pairs of compounds, 30 with 46 and 51 with 55. The molecular shape may be

expected to have a profound effect on physical properties, and thus to influence such factors as lipid/water partition and membrane permeability.

As regards the chemical reactivity of the aminonitriles, their behaviour on hydrolysis is of possible significance. This can take two pathways



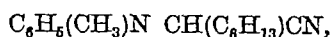
and liberation of cyanide in the biophase must be considered as having a possible bearing on their mode of action. We do not believe, however, that this is the ultimate mechanism of the insecticidal activity for the following reasons. Phenylmethylaminooctonic nitrile (no. 17) causes 80% absolute mortality of chrysanthemum aphids at 0.2%. This is equivalent to a concentration of approximately 0.02% HCN. With compound no. 50, producing 98% absolute mortality at 0.025%, the equivalent HCN concentration would be only 0.004%. Actual experiment showed that sodium cyanide was practically non-toxic to chrysanthemum aphids at 0.04%. This evidence is reinforced by the lack of aphicidal activity in heptaldehyde cyanohydrin, a fat-soluble compound which can also give rise to cyanide on decomposition.

It is our view that the insecticidal activity of the α aminonitriles discussed in this paper is to be attributed partly to their chemical reactivity which enables them to become attached to cell constituents. This may be through the unsaturated nature of the cyano group, as in thiocyanates. Alternatively, it may be due to a displacement reaction whereby some group such as NH_2 in the cell displaces the group R_2N from the aminonitrile. We have, indeed, observed that heating $(\text{C}_6\text{H}_5)_2\text{N} \text{ CH}(\text{C}_6\text{H}_5)_2\text{CN}$ with aniline causes evolution of diethylamine. Superimposed on this reactivity and indeed dominating the entire situation, is the effect of the physicochemical properties of the molecule as a whole, the correct adjustment of such factors as lipid/water partition, molecular shape, surface activity, etc., determining the absorption of the insecticide and its distribution within the organism. This parallels the case of the thiocyanates, and conforms to the general views regarding contact insecticidal action put forward by Luger, Martin & Muller (1944) and further illustrated by Davies & Sexton (1948).

SUMMARY

1 The insecticidal properties of a series of substituted α aminonitriles have been examined, and marked activity found in certain compounds.

2 There is marked specificity in the resistance of different insects to individual compounds.

3 α -Phenylmethylaninoctonic nitrile,and $\alpha\beta$ bis(α -cyano α -heptylamino)ethane,

have aphicidal activity only slightly less than that of dodecyl thiocyanate or nicotine

4 The mode of action of these insecticides is discussed in relation to their chemical reactivity and their physicochemical properties

We wish to thank the entomologists of Hawthornedale Laboratories, Jealott's Hill Research Station, and particularly Mr F J D Thomas, for carrying out the biological testing. We are indebted also to Dr B Collie for the preparation of emulsions

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The Occurrence of Phenolic Substances in Arthropods

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Pryor (1940) showed that the hardening of the protein component of insect cuticle is due to the tanning action of an agent produced by oxidation of a phenolic substance. In the analogous hardening of the cockroach ootheca, Pryor, Russell & Todd (1946) showed that the phenolic substance concerned is 3,4-dihydroxybenzoic acid (I) and suggested a possible mechanism for the hardening process. It was clearly desirable to examine a number of insect species in order to establish the range of phenolic substances which might be concerned.

Polyhydric phenols appear to be widely distributed in arthropods, although very few have actually been isolated, most investigators having simply demonstrated their presence in ethanolic extracts by means of colour reactions. In this way, Pryor (1940) has shown that *o*-dihydric phenols occur in representatives of six orders of insects (Orthoptera, Odonata, Coleoptera, Hemiptera, Lepidoptera, Hymenoptera), and Lafon (1943) has found them in insects and in Arachnida (Scorpionidea, Araneida). In insects, phenols are associated with some of the layers of the epicuticle (Wigglesworth, 1947, Webb, 1947) and with the brown proteins (sclerotins) which are responsible for the hardness of the exocuticle. They also occur in the epicuticle of

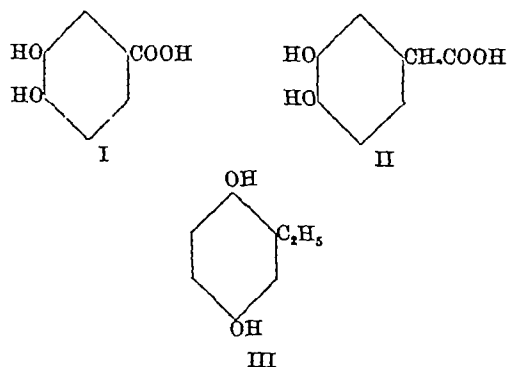
arthropods, whose exocuticle is hardened with calcium carbonate, by a less specific test (the reduction of ammoniacal silver oxide, performed on paraffin sections) we have shown that they are probably present in the epicuticle of a typical diplopod (*Tachypodorus niger* Leach), and Dennell (1947b) has found them in the epicuticle of several species of Crustacea Malacostraca. Using the same test, Beament (1947) has found that some of the layers of the chorion of the eggs of *Rhodnius prolixus* Stahl (Hemiptera, Reduviidae) are rich in dihydric phenols.

The earliest recorded isolation of a phenolic substance from insects is that of Villon, who is reported by Slater (1887) as having obtained 15 g of an 'alcohol soluble tannin' from 500 g of *Sitophilus granarius* L. Schmalfuss & Muller (1927) isolated 3,4-dihydroxyphenylalanine from the elytra of two species of *Melolontha*. Schmalfuss, Heider & Winkelmann (1933) isolated 3,4-dihydroxyphenylacetic acid (II) from the elytra of *Tenebrio molitor* L., and the same acid was obtained by Schmalfuss (1937) from the cuticles of *Cetonia aurata* L., *Potosia cuprea* F. and *Melolontha hippocastani* F. (Coleoptera, Scarabaeidae). More recently, Pryor *et al* (1946) isolated 3,4-dihydroxybenzoic acid (protocatechuic

acid) (I) from oothecae of *Blatta orientalis*, and the same authors (Pryor *et al* 1947) obtained 3,4-dihydroxybenzoic acid from puparia of *Calliphora erythrocephala* L and 3,4-dihydroxyphenylacetic acid and α -hydroxy- β -(3,4-dihydroxyphenyl) propionic acid (3,4-dihydroxyphenyllactic acid) from imagines of *Tenebrio molitor* L.

We have now examined the following species: *Locusta migratoria* L., *Rhizopertha dominica* F (Coleoptera, Bostrychidae), *Sitophilus* (= *Calandra auct non Clairv*) *granarius* L., *S. oryzae* L (Coleoptera, Curculionidae), *Tribolium confusum* J du Val and *T. ferrugineum* F (= *castaneum* Herbst) (Coleoptera, Tenebrionidae).

The experimental procedure was a modified version of that used in our previous studies and gave much higher yields of phenolic acids. The modifications included freeze drying of the insect material, followed by mechanical grinding, as well as minor changes in the methods of purification. During the isolation procedure, the phenolic acids present were methylated to facilitate working up, so that the acids were actually isolated as their methyl ethers. Previous work (Pryor *et al* 1946, 1947) leaves no reasonable doubt that the acids present in the insects are in the unmethylated form. The results, which are recorded in the experimental portion, show that all the species examined contain 3,4-dihydroxyphenylacetic acid (II) and the *Tribolium* species contain, in addition, 3,4-dihydroxybenzoic acid (I). The low yield of 3,4-dimethoxyphenylacetic acid obtained from *Locusta* is perhaps to be ascribed to the low ratio of cuticle weight to total weight, as compared with the ratio in the other insects investigated. In addition to the insects mentioned in the experimental section we also re-examined imagines of *Tenebrio molitor*, but, in confirmation of our earlier results (Pryor *et al* 1947), we were unable to detect in them any 3,4-dihydroxybenzoic acid.



Summarizing the information available from the present and earlier work, it appears that phenolic acids have been identified by isolation (as such or as their methyl ethers) from thirteen representative

species belonging to three orders of Insecta, whilst colour reactions indicate the presence of similar *o*-dihydroxyphenolic substances in four additional orders. We feel justified, therefore, in assuming that these phenolic acids are of general occurrence and may all be concerned in cuticle hardening. Although the presence of traces of acids other than those identified cannot be wholly excluded, the pure methylated substances isolated by us accounted for more than 75% of the crude methylated phenolic extracts, so that the distribution of the identified acids is of some interest. Of the thirteen species so far examined, ten contained 3,4-dihydroxyphenylacetic acid, which in seven cases was the only acid present, in two cases it was accompanied by 3,4-dihydroxybenzoic acid, and in one by α -hydroxy- β -(3,4-dihydroxyphenyl) propionic acid. 3,4-Dihydroxybenzoic acid occurred alone in two species. In addition, 3,4-dihydroxyphenylalanine has been isolated from two species. The most likely mode of participation of such *o*-dihydroxy aromatic acids in cuticle hardening is enzymic oxidation followed by condensation of the oxidized material with free amino groups in the cuticular protein, so that stable cross-linked structures are formed in which the nitrogen of the amino groups becomes directly attached to the aromatic nuclei. Thiol groups in the protein may, of course, also take part in reaction with the oxidized dihydroxy acid. The process, in deed, bears a close resemblance to the tanning of leather with quinones. Catechol and 3,4-dihydroxybenzoic acid and their homologues when oxidized condense *in vitro* with compounds containing free amino groups to give compounds in which nitrogen is directly attached to the aromatic nucleus. Reactions of this type are being studied at present as an aspect of the work here described and will be reported upon in due course. As to the origin of the phenolic acids in insects, the most probable explanation is that they are degradation products of 3,4-dihydroxyphenylalanine, itself produced from tyrosine under the influence of a polyphenol oxidase. It is of interest to note that both Fraenkel & Rudall (1947) and Dennell (1947a) have also expressed the view that the phenol hardening in blowfly puparia is brought about by a phenolic material produced from blood tyrosine by enzymic oxidation.

Since it was desired to make our investigations as comprehensive as possible, the solutions of sulphurous acid containing cyanide in which the insects were killed and stored were always examined for the presence of phenolic substances. In the case of the two *Tribolium* species, but in no others, ether extracts of the acidic liquor, after removal of the dead insects, yielded large quantities of ethylhydroquinone (1.4% on freeze-dried insect material) identified by analysis, mixed melting point and preparation of the *dibenzoate*. This substantially con-

firmes the findings of Alexander & Barton (1943), who examined the volatile secretion of the flour beetles, *Tribolium confusum* J du Val and *T ferrugineum* F (= *castaneum* Herbst), which causes flour infested by these beetles to become pink in colour and unpalatable, they concluded on the evidence available to them that the active principle was ethyl *p*-benzoquinone. Presumably in our experiments the insects, on being dropped into the sulphurous acid solution, eject ethyl *p*-benzoquinone which is then reduced to the hydroquinone. Quinones have previously been recorded in secretions of the stink glands of other arthropods. Béhal & Physalix (1900) described a compound with the properties of a *p*-quinone in the lateral stink glands of the millipede, *Schizophyllum mediterraneum* Latzel, but did not isolate it, similar observations on other diplopods are described by Burt (1947). Ethyl *p*-benzoquinone is probably also the chief constituent of the yellow oils obtained by Palm (1946) from stink glands on the prothorax and on the ventral side of the tip of the abdomen of *Aphanotus destructor* Uytt and *Tribolium confusum* J du Val.

The crude crystalline material first obtained by us during the isolation of ethylhydroquinone had a low melting point and was difficult to purify. Careful fractionation of crystallization mother liquors obtained in purifying the ethylhydroquinone and its dibenzoate yielded products which, although melting lower than methylhydroquinone (toluhydroquinone) and its dibenzoate, showed no depression in melting point when mixed with authentic specimens of these materials, but were depressed in melting point by pure ethylhydroquinone and its dibenzoate. From these facts and the elementary analysis, we conclude that the extract contained small amounts of toluhydroquinone. It is probable that the similar difficulty experienced by Alexander & Barton (1943) in purifying their material was due to the presence in the volatile secretion of small amounts of methyl *p*-benzoquinone (toluquinone) as well as ethyl *p*-benzoquinone.

The most probable precursor of these *p*-quinones would seem to be 2,5-dihydroxyphenylalanine, an acid already suggested as an intermediate in the formation of homogentisic acid (2,5-dihydroxyphenylacetic acid) in human alcaptonuria (Neuberger, Rimington & Wilson, 1947). 2,5-Dihydroxyphenylalanine itself has not been found in nature, but its production from tyrosine (cf Blaschko & Sloane Stanley, 1948) by oxidation as an alternative to 3,4-dihydroxyphenylalanine would seem reasonable enough. Such an oxidation would be analogous to the well-known formation of toluhydroquinone on oxidizing *p*-cresol with potassium persulphate in acid solution, rearrangement with migration of the methyl group occurring in the process (Kumogi & Wolfenstein, 1908), a similar

migration of the aminopropionic acid side chain during the oxidation of tyrosine would yield 2,5-dihydroxyphenylalanine, which might then be degraded to ethylhydroquinone and toluhydroquinone. Wolkow & Baumann (1891) have already shown that the intestinal bacteria in dogs can convert homogentisic acid to toluhydroquinone.

EXPERIMENTAL

Methylated phenolic acids from insects

The insects were collected and stored in water containing KCN to inactivate polyphenol oxidases, and SO₂ to prevent aerial oxidation of hydroquinone or catechol derivatives. Before working up, the material (whole insects, except in the case of *Locusta* where only the heads were used) was separated from the liquid, freeze dried and ground in a mechanical mortar. It was then extracted with methanol in a vapour jacketed Soxhlet for 24 hr, filtered, the methanolic extracts (which had a blue fluorescence) evaporated to dryness under reduced pressure in an atmosphere of N₂, and the residue extracted with water until the extracts gave a negative reaction with FeCl₃. The combined aqueous extracts were acidified to congo red with H₂SO₄, extracted continuously with peroxide free ether, the ethereal extract concentrated to c. 100 ml and the phenols present methylated by addition of ethereal diazomethane (from 20 g nitrosomethylurea). The solution so obtained was left overnight, solvents removed by evaporation and the residual oil refluxed for 2 hr with methanolic KOH (50 ml of 2%) to hydrolyze ester groups. After removal of methanol, water was added, the solution acidified with H₂SO₄ and extracted with ether. The ethereal extract was shaken with aqueous Na₂CO₃ to remove acids, and the aqueous layer was again acidified and extracted with ether. The ethereal extract dried over Na₂SO₄ and evaporated yielded usually a semi-solid mass. Fractional crystallization, or, where only one acid was present, simple recrystallization, from a mixture of benzene and light petroleum yielded the pure methylated acids. The products obtained in each case are given below.

Locusta migratoria 3,4-Dimethoxyphenylacetic acid, m.p. 97°, undepressed by an authentic specimen, m.p. 98° (Found C, 61.3, H, 6.4. Calc. for C₁₀H₁₂O₄, C, 61.2, H, 6.2%). Yield, 0.2% on dried material (415 g).

Rhizopertha dominica 3,4-Dimethoxyphenylacetic acid, m.p. and mixed m.p. 98° (Found C, 61.9, H, 6.0%). Yield, 1.5% on dried material (30 g).

Strophilus granarius 3,4-Dimethoxyphenylacetic acid, m.p. and mixed m.p. 96° (Found C, 61.3, H, 6.0%). Yield, 1.8% on dried material (101 g).

S. oryzae 3,4-Dimethoxyphenylacetic acid, m.p. and mixed m.p. 97° (Found C, 60.9, H, 5.9%). Yield, 1.4% on dried material (128 g).

Tribolium confusum 3,4-Dimethoxyphenylacetic acid (yield, 1.4%), m.p. and mixed m.p. 96° (Found C, 61.1, H, 6.4%), together with 3,4-dimethoxybenzoic acid (yield, 0.4%), m.p. 179°, undepressed by an authentic specimen, m.p. 180° (Found C, 59.3, H, 5.7. Calc. for C₉H₁₀O₄, C, 59.3, H, 5.5%). The amount of dried insects used was 45 g.

T. ferrugineum 3,4-Dimethoxyphenylacetic acid (yield, 1.9%), m.p. and mixed m.p. 98° (Found C, 61.0, H, 5.9%),

together with 3 4-dimethoxybenzoic acid (yield, 0.5%), m p and mixed m p 180° (Found C, 59.1, H, 5.6%) The amount of dried insects used was 57 g

Isolation of ethylhydroquinone (1 4-dihydroxy-2 ethylbenzene)

The acidic liquid from the *Tribolium* species, after separation of the insect bodies (dry wt 100 g), was continuously extracted with ether for 24 hr, and the ethereal extract dried over Na_2SO_4 and evaporated. The residual oil was now treated with an excess of concentrated aqueous FeCl_3 solution and quinones formed were collected by steam distillation. The steam distillate was saturated with SO_2 and extracted with ether. Evaporation of the ethereal extract gave the crude ethylhydroquinone as a colourless crystalline solid, m p 106°, unaltered by repeated recrystallization from benzene or chloroform. After several recrystallizations from water (charcoal) the product formed colourless plates, m p 114°, alone or in admixture with authentic ethylhydroquinone (m p 114°) prepared by Clemmensen reduction of 2 5 dihydroxyacetophenone (Found C, 69.9, H, 7.5. Calc for $\text{C}_8\text{H}_{10}\text{O}_2$, C, 69.6, H, 7.3%). For further confirmation of its identity, the product was treated with excess of benzoyl chloride in pyridine. The dibenzoate so formed separated from methanol in colourless needles, m p 89°, undepressed in admixture with authentic dibenzoate of ethylhydroquinone (m p 89°) prepared as described below (Found C, 76.1, H, 5.0. Calc for $\text{C}_{22}\text{H}_{18}\text{O}_4$, C, 76.2, H, 5.2%).

Concentration of the aqueous crystallization mother liquors of the ethylhydroquinone yielded a crop of material which, after further fractionation from water and recrystallization from benzene, had m p 114°, undepressed in admixture with toluhydroquinone (m p 124°), but depressed in admixture with ethylhydroquinone (m p 114°). Similarly, fractional crystallization from methanol of the dibenzoate prepared from the crude ethylhydroquinone, yielded a small amount of material, m p 93°, undepressed in admixture with the dibenzoate of toluhydroquinone

(m p 122°), but depressed in admixture with the dibenzoate of ethylhydroquinone (m p 89°) (Found C, 76.1, H, 5.1. Calc for $\text{C}_{21}\text{H}_{16}\text{O}_4$, C, 75.9, H, 4.85. Calc for $\text{C}_{22}\text{H}_{18}\text{O}_4$, C, 76.2, H, 5.2%).

Ethylhydroquinone dibenzoate Benzoyl chloride (1 ml.) was added to a solution of ethylhydroquinone (0.5 g) in dry pyridine (10 ml). Reaction set in at once and the mixture was allowed to stand overnight and then poured on a mixture of ice and dilute H_2SO_4 and extracted with ether. The ethereal extract was thoroughly washed, dried over Na_2SO_4 and evaporated. Recrystallization of the solid residue from methanol (charcoal) gave *ethylhydroquinone dibenzoate* as colourless needles, m p 89° (Found C, 76.2, H, 5.3. $\text{C}_{22}\text{H}_{18}\text{O}_4$ requires C, 76.2, H, 5.2%).

SUMMARY

1 The occurrence of 3 4-dihydroxyphenylacetic acid in six insect species is reported. Two of these species contain, in addition, 3 4 dihydroxybenzoic acid. These phenolic acids are believed to play a part in the hardening of insect cuticle.

2 From aqueous sulphurous acid used to kill and store two species of *Tribolium*, ethylhydroquinone has been isolated in substantial yield. Its isolation confirms the results of earlier workers who showed that ethyl *p* benzoquinone is secreted by these insects. Evidence is presented that toluhydroquinone is also present in the extracts.

We record our gratitude to the Ramsay Memorial Trustees for a Fellowship and to the Council for Scientific and Industrial Research (Australia) for a Studentship award to one of us (R. H. H.). We are also very much indebted to Dr E. A. Parking of the Pest Infestation Laboratory, Slough, and to Monsieur Chaboussou, Director of the Station de Zoologie Agricole du Sud Ouest, Bordeaux, for their assistance in collecting material.

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The Effect of High Blood Urea on the Acid-base Balance of Serum of Dogs

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(Received 31 March 1948)

In studies on the changes of electrolyte excretion in unilateral tartrate nephrosis and on the added effect of removal of the normal kidney on the acid base balance, it was thought advisable to determine the effect of high blood urea values on the acid-base balance of the blood, because possibly the changes in osmotic equilibrium brought about by the increase in urea might change the electrolyte balance. Streicher (1928) found that injections of 10–20% urea solutions in sufficient amounts to raise the blood urea nitrogen of dogs to as high as 328 mg urea N/100 ml produced drops in the CO_2 content of the plasma of from 15.3 to 20.8 ml/100 ml of plasma, the CO_2 estimations being made when the blood urea was from 14 to 130 mg/100 ml above the normal level. He also reported that the blood chloride and calcium were raised and the potassium lowered.

EXPERIMENTAL PROCEDURE

Dogs weighing from 12 to 19 kg were kept in metabolism cages during the period of the experiment and were fed their usual mixed diet. Water was given *ad lib*, the amount taken being measured, and the total fluid output collected and measured. The urea, non protein nitrogen, total base, CO_2 , Cl, inorganic phosphate and protein of serum were determined, immediately before and at varying intervals after the administration of the urea solutions. The blood was obtained from the femoral vein without stasis, with drawn without contact with air, placed under oil in tubes with constricted necks and centrifuged.

The urea was administered intravenously in 33.3% aqueous solution at 38° and at the rate of 2 ml/min as described by Leiter (1921). In most dogs the first blood sample was drawn at 10 a.m., the urea given at 10.20 a.m. and subsequent blood samples drawn for analysis at 11.20 a.m. and 4.45 p.m. of the same day. A few experiments were carried on for 3 days. In these the urea was administered at 9.20 a.m. and 4.30 p.m. on the first day, 4.30 p.m. on the second day and at 1.45 p.m. on the third day. Blood samples were drawn at 9 a.m. and 2 p.m. on the first day, at 9 a.m. on the second day and at 9 a.m. (for blood urea estimation only) and at 4.30 p.m. on the third day.

Carbon dioxide was determined by the manometric method of Van Slyke & Neill (1924), chlorides in tungstic acid filtrates by the titrimetric method of Sendroy (1937), and phosphates by the method of King (1932). The serum protein was calculated by subtracting the figure for non-

protein nitrogen, estimated by the method of Folin & Wu (1919), from the total nitrogen determined by the usual Kjeldahl procedure using 0.5 ml. of serum. The usual factor of 6.25 was used to convert the figure for protein nitrogen to protein. The method of Van Slyke & Cullen (1914) was used for urea. The total base estimations were done by the method of Stadie & Ross (1925). In the last four experiments determinations were also done by electro dialysis (Consolazio & Talbott, 1940). All determinations were done in triplicate.

Calculations. The CO_2 values were converted from ml/100 ml serum to mmol/l by the formula

$$B = \text{CO}_2 - \frac{14.04 \text{ CO}_2}{\text{antilog}(\text{pH} - 6.1)} \times 0.713, \\ 2.24$$

as given by Peters, Bulger, Eisenman & Lee (1926). The phosphate in mg P/100 ml serum was converted to mmol/l by the factor 18/31.04 as given by the above authors. The base combining properties of the protein as m equiv were calculated from the formulae of Van Slyke, Hastings, Hiller & Sendroy (1928). $B.P. = 1.072 \times \text{g P} (\text{pH} - 5.04) (A/G = 1.8)$. In four experiments the pH was determined with the Beckman enclosed glass electrode, in the remainder, a pH of 7.35 was assumed. Although the pH rose during these experiments the maximum error involved by assuming pH 7.35 would be not more than 2 m equiv, the error in all instances tending to a lowering of the acid column.

RESULTS

Twelve dogs were used. All the dogs remained moderately well during the course of the experiments. There was a marked diuresis in all of them with occasional vomiting and slight diarrhoea in three animals, the results from these three were discarded. The total fluid output of all dogs was slightly less than the total intake. There was no blood in the faeces, and no convulsions occurred. The serum urea rose to levels of 320–590 mg/100 ml serum 1 hr from the administration of the urea solution and fell to levels of 160–360 mg by 4.45 p.m. on the same day. In the 3 day experiments an occasional drop to near the normal level was noted by the morning of the third day.

The results of four typical experiments are shown in Table 1. There was a rise in the pH of the serum varying from 0.03 to 0.08. The effect on the total base and on the total determined acid, as well as the individual constituents of the acid column, was

Table 1 *The changes in acid base balance accompanying high blood urea levels produced by urea injections*

Example no	Wt of dog (kg)	Time	pH	Phos phate	Chloride	Protein	CO ₂	Total acid	Total base	Urea (mg / 100 ml)	Urea admin (g)
				(mmol / l)							
1	19	10 00 a m	—	3 1	102 8	16 3	23 9	146 1	152 7	15 0	—
		10 20 a m	—	—	—	—	—	—	—	—	95
		11.20 a m	—	3 8	102 8	21 8	27 5	155 9	152 2	—	—
		4 45 p m	—	3 4	102 8	17 5	25 9	149 6	151 0	—	—
2	17	10 00 a m	—	3 7	100 8	16 6	23 9	145 0	147 4	—	—
		10 20 a m	—	—	—	—	—	—	—	—	08
		11 20 a m	—	4 3	102 5	17 5	25 6	149 9	146 3	540	—
		4 45 p m	—	4 6	107 0	18 3	23 7	153 6	157 6	332	—
3	16	10 00 a m	7 36	3 5	100 6	14 9	24 5	143 5	153 5	19	—
									154 1*		
		10 20 a m	—	—	—	—	—	—	—	—	64
		11 20 a m	7 43	4 2	101 1	15 8	26 2	147 3	150 0	486	—
									158 2*		
4	12	4 45 p m	7 40	4 2	104 2	18 8	25 5	152 7	149 0	368	—
									161 9*		
		9 00 a m	7 35	2 6	108 0	16 1	22 6	149 3	156 6	40	—
									155 9*		
		9 20 a m	—	—	—	—	—	—	—	—	48
		2 00 p m	7 41	3 6	108 0	16 1	23 3	151 6	155 6	321	—
									162 7*		
		4.30 p m	—	—	—	—	—	—	—	—	24
		9 00 a m	7 39	2 3	109 7	12 9	22 7	147 6	148 9	326	—
									160 1*		
		4 30 p m	—	—	—	—	—	—	—	—	48
		9 00 a m	—	—	—	—	—	—	—	58	—
		1 45 p m	—	—	—	—	—	—	—	—	48
		4 30 p m	7 42	2 6	104 1	14 6	23 4	144 7	140 6	311	—
										151 9*	

* Total base determined by electro dialysis (Consolazio & Talbott, 1940)

somewhat variable, but some pronounced general tendencies were noted. In the short experiments there was an invariable absolute increase in the total determined acids which was maintained throughout the experimental period in all but one instance. Even in this case (Example 1), however, the determined acids were slightly higher at the end than at the beginning of the experiment. In the first hour there was a definite rise in the phosphate, protein and CO₂ and in all but one experiment (Example 1) the chloride. Between the taking of the second blood sample, 1 hr after the injection of the urea solution, and the taking of the last specimen, 6½ hr afterwards, the CO₂ values usually fell slightly but, as a rule, they did not return to the original level in this time. The protein values usually rose still further in this period, but occasionally a small drop was noted. In all but one instance (Example 1) the chlorides increased more than enough to offset any decreases of CO₂ or protein.

There was a significant effect on the total base. When determined after incineration the total base was always lowered either absolutely or in relation to the sum of the determined acids, the value for the latter often exceeding the total inorganic base. When the total base was determined by electro dialysis,

however, it was found that the usual excess of total base over the sum of the determined acids existed (Examples 3 and 4).

DISCUSSION

The difference between the base, as determined after incineration, and the figure obtained by electro dialysis could be accounted for only by the presence of some organic base in the blood. The nature of the extra base has not been determined. That it cannot be ammonia seems certain because the differences observed were too great to be accounted for by any increase in blood ammonia which could be tolerated by the animals, and in one animal in which the blood ammonia was determined no increase was observed. As the urea solution was neutral in reaction and gave no base on electro dialysis the organic base must have been either produced in the animal or have diffused from the tissues into the blood. The observed changes in inorganic base and in the serum anions may be best explained in relation to the presence of this extra base in the blood. Occasional observations of excess of acid over base in the serum have been reported (Peters, Wakeman, Eisenman & Lee, 1929, Atchley & Benedict, 1930, Sunderman, 1931, Hald, 1933). The instances recorded by the first three

groups of authors (Peters *et al* , Atchley & Benedict, Sunderman) all occurred in individuals with low total base in a variety of conditions—nephritis, diabetic acidosis, pneumonia—while Hald reported the occurrence of an apparent acid excess in some normal individuals. Hald could find no reasonable explanation for the seeming anomaly but as our findings offer reasonable presumptive evidence of the occurrence of an organic base in the blood under one set of experimental conditions, it may be assumed that such base could occur in other conditions. The nature of the base and the mechanisms responsible for its appearance in the blood remain to be determined.

SUMMARY

1 The effect of increases in blood urea produced by the intravenous injection of urea solutions on the electrolyte pattern of the serum was studied

2 There was an increase in the amounts of the determined acids and a decrease in the amount of total base determined after incineration

3 When the total base was also determined by electro dialysis no such decrease was found

4 The difference between the figures for total base obtained by the two methods is taken as reasonable presumptive evidence for the presence of some organic base in the animals with a high blood urea

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The 269th Meeting of the Biochemical Society was held in the Department of Biochemistry, The University, Sheffield 10, on Friday, 24 September 1948, at 11.30 a.m., when the following papers were read

COMMUNICATIONS

Observations on the Type of Hydroxyl Group undergoing the Ethereal Sulphate Synthesis in Rabbits By J. I. ANDERTON, J. N. SMITH and R. T. WILLIAMS (Department of Biochemistry, University of Liverpool)

It is generally believed that the only hydroxy compounds which form ethereal sulphates on administration to animals are phenols. Prof. G. F. Marrian (personal communication) has drawn our attention to the fact that a number of ethereal sulphates of non-phenolic steroids have been isolated from normal and pathological urines, e.g. androsterone-3 sulphate (Venning, Hoffman & Browne, 1942). In these steroids the sulphated hydroxyl group is not phenolic but occurs in a secondary alcohol group, >CHOH . The production of androsterone 3 sulphate by administration of androsterone itself has not been studied. There is therefore no proof as yet that androsterone combines directly with sulphate *in vivo*. It has been suggested (Dodgson, Garton, Stubbs & Williams, 1948) that the ability of a hydroxy compound to form an ethereal sulphate may depend on its ionization constant. A survey of phenols which are known to conjugate with sulphate shows that the $\text{pK}'\text{s}$ of the OH groups vary from c. 7 to 10. We therefore decided to survey ethereal sulphate formation on feeding a large number of hydroxy compounds and compare the results with known values of the ionization constants of the hydroxyl groups. The results quoted below are but preliminary observations.

Some of the pyrimidines quoted above, e.g. veronal, uracil, alloxan and isobarbituric acid, possess $\text{pK}'\text{s}$ of the same order as phenols, but only the last forms an ethereal sulphate (also in dogs, Cerecedo, 1930). If the formulae of these pyrimidines are examined, it will be found that only

isobarbituric acid possesses an OH in the system, $\text{C}=\text{C}(\text{OH})-\text{C}$, the others contain the system $-\text{N}=\text{C}(\text{OH})-\text{C}$ (cf. the hydroxypyridines). Our results suggest two conditions for ethereal sulphate

Compound	% of dose excreted as sulphate in rabbits	pK of OH group
Barbituric acid	0	4.0
Diethylbarbituric acid (veronal)	0	7.4
isoBarbituric acid (hydroxyuracil)	11	8.6
Uracil	0	9.45
Alloxan	0	6.6
Theobromine	0	9.9
Ascorbic acid	0	4.2 and 11.6
Ethanol	0	> 15.0
β Hydroxypyridine	29	? (phenolic)
α Hydroxypyridine (α pyridone)	0	? (not phenolic)
Compare with, for example		
Phenol	19	10
Catechol	18	9.5
Resorcinol	13	9.8
<i>p</i> Chlorophenol	22	9.2
<i>o</i> Nitrophenol	7	7.25
<i>p</i> Hydroxybenzoic acid	7	9.4

formation (a) a pK , c. 7–10 and (b) the OH in an enolic system, $\text{C}=\text{C}(\text{OH})-\text{C}$. Ascorbic acid contains this system, i.e. $\text{C}-\text{C}(\text{OH})=\text{C}(\text{OH})-\text{C}$, but it forms no ethereal sulphate for the $\text{pK}'\text{s}$ of the OH groups are respectively less than 7 and greater than 10. It is also significant that the enzyme phenolsulphatase only hydrolyzes phenolic-ethereal sulphates but not alcoholic sulphates such as ethylsulphuric and amylsulphuric acids (see summary in Fromageot, 1938).

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The Decarboxylation of *ortho*-Tyrosine (*o*-Hydroxyphenylalanine) By H. BLASCHKO and G. H. SLOANE STANLEY (*Department of Pharmacology, University of Oxford*)

meta Tyrosine has been shown to be a substrate of the mammalian enzyme dopa decarboxylase (Blaschko & Sloane Stanley, 1948). The amino acid was not decarboxylated by intact resting cells of *Streptococcus faecalis* R, but it has since been found that it is decarboxylated by acetone dried preparations of the bacteria at about one third of the rate at which L tyrosine is decarboxylated.

We have now examined *ortho*-tyrosine. This amino acid was found to be decarboxylated by mammalian tissue extracts (guinea pig's kidney and rat's liver). The total amount of CO₂ formed suggests

that only the L isomer was attacked. Competition experiments showed that the same catalyst was responsible for the decarboxylation of both *meta* and *ortho*-tyrosine. No significant decarboxylation occurred with acetone dried preparations of *Strep faecalis* R.

ortho Tyrosine was oxidized by the D amino acid oxidase of pig's kidney and by the L amino acid oxidase of cobra venom.

We are grateful to Dr A. Neuberger for the sample of DL-*ortho* tyrosine used in these experiments.

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Mild Acid Hydrolysis of Human Blood Group A-substance By D. AMINOFF, W. T. J. MORGAN and WINIFRED M. WATKINS (*Lister Institute of Preventive Medicine, London*)

The human blood group A substance shows two distinct serological characters both of which have been used to measure the amount of A substance. The more labile character, that of inhibiting the agglutination of A erythrocytes by human or animal anti-A sera, is a sensitive indicator of any structural changes brought about by the isolation processes. The second serological property, that of inhibiting the haemolysis of sheep red cells by rabbit anti-A serum and complement, is relatively stable and is susceptible of more accurate assay. The serological properties of the A substance are not destroyed in solution at R.T. between pH 3.5 and 7.0. Heating with N acetic acid at 100° for 18 hr, however, brings about almost complete inactivation as measured by the power of the A-substance to inhibit agglutination and at the same time gives rise to a material which shows an enhanced capacity to inhibit sheep cell haemolysis.

During the hydrolysis the reducing power of the A substance, expressed as glucose, increases from 1% or less to about 10%. The reddish purple colour developed on treating the solution with 0.05N-Na₂CO₃ at 100° for a few minutes followed by the addition of *p* dimethylaminobenzaldehyde HCl reagent, increases from an initial value of 10% to a steady value equivalent to about 13% of N acetylglucosamine. After 18 hr heating the hydrolysis products can be separated into a material (38% by weight) which diffuses through a Cellophane membrane (see Morgan, 1946) and an indiffusible moiety. The latter material shows no significant power to inhibit agglutination but possesses a five fold increase in activity, on a weight basis, and

measured by the haemolysis inhibition test. The diffusible material, without further hydrolysis, gives 21% reduction (as glucose), contains 3.4% N and develops a colour, after treatment with 0.05N-Na₂CO₃ and Ehrlich's reagent, equivalent to 6% N acetylglucosamine. The material retained by the membrane shows a dextro rotation (α)_D 54.61 ± 5.1 ± 6°, possesses a small reducing power only (2.5%), contains 6.6% N and gives a colour reaction for N acetylhexosamine equivalent to 17% N-acetylglucosamine.

The diffusible (a) and indiffusible (b) materials after hydrolysis with 0.5N or 6N-HCl yield maximum values for reduction (a, 32%, b, 46%), hexosamine (a, 16%, b, 39%) and amino acid N (a, 0.6%, b, 2.1%). There is no sharp separation of the two amino sugars believed to be present in the A substance (Aminoff & Morgan, 1948) into one of the fractions. Chromatographic examination reveals that the major part of the L-fucose passes into the diffusate thus indicating that this component is readily removed from the A-mucoid and is therefore not intimately associated with the structure responsible for the serological property of inhibiting sheep cell haemolysis. The diffusible components appear to be free from leucine and isoleucine and possibly from proline, and to contain a small fraction only of the total hydroxyamino acids.

The undegraded A-substance gives no reaction with pneumococcus Type XIV horse serum whereas the indiffusible material precipitates slightly with this immune-body (see also Kabat, Baer, Bezer & Knaub, 1948).

It is believed that a closer study of the essential constituent in the indiffusible material which is responsible for inhibiting the haemolytic action of the anti-A serum, will reveal the nature of the

structure associated with this property and thus provide a useful approach to the larger problems of the nature of heterogenetic antigens and haemolysis by immune serum

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Volatile Metabolic Products of *Endoconidiophora coerulea* Munch By E N MORGAN (introduced by J H BIRKINSHAW) (*Division of Biochemistry, London School of Hygiene and Tropical Medicine*)

The fungus *Endoconidiophora coerulea*, one of the agents causing 'blue stain' in sapwood of coniferous timbers, produces in culture an odour resembling that of an ester of one of the higher alcohols, e.g. amyl acetate. The work now described was undertaken in order to identify the odorous constituents.

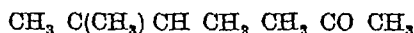
The fungus was inoculated into ninety-two flasks each containing 350 ml of aqueous malt extract (4%) and incubated at 24° for 15 days. The volatile products were obtained by steam distillation of the total flask contents, followed by ether extraction of the aqueous distillate. After removal of ether the colourless residual oil was subjected to distillation. Two main fractions, both liquid, were thus obtained.

Fraction I b.p. 110–120°/764 mm, 0.65 g
 Fraction II b.p. 173–176°/764 mm, 4.48 g

Fraction I proved to be isobutyl acetate, since on hydrolysis it afforded isobutyl alcohol and acetic acid characterized as the 3,5-dinitrobenzoate and the *p*-bromophenacyl ester respectively.

Fraction II consisted in part of an unsaturated carbonyl compound, C₈H₁₄O. It gave characteristic

crystalline derivatives, namely, the 2,4-dinitrophenylhydrazone (orange needles, m.p. 87°) and the semicarbazone (needles, m.p. 135–136°). Evidence as to the constitution of this product was obtained by degradation with ozone and oxidation with KMnO₄-CrO₃. Ozonization in chloroform gave rise to acetone, laevulaldehyde, and methyl glyoxal (cf. Pummerer, Matthäus & Socas-Viñals, 1936), whilst oxidation with KMnO₄ followed by CrO₃ (Tiemann & Semmler, 1895) gave acetone and laevulinic acid. These reactions indicated that the product was 2-methylhept-2-en-6-one,



When the dinitrophenylhydrazone and the semicarbazone of the fungal metabolic product were compared with the corresponding derivatives of authentic naturally occurring methylheptenone they were found to have the same melting point and gave no depression on mixing.

The odour of methylheptenone has been described as resembling that of amyl acetate. The two volatile products isolated, isobutyl acetate and methylheptenone, are probably the main contributors to the odour of the fungus in culture.

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The Elimination of Methylthiol and Dimethyl Sulphide from Methylthiol- and Dimethylsulphonium compounds by Moulds By FREDERICK CHALLENGER and Y C LIU (*Department of Organic Chemistry, The University, Leeds 2*)

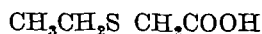
Methionine and *S*-methylcysteine in bread cultures of *Scopulariopsis brevicaulis* Saccardo (Strain Washington 2, no. 580 Nat. Coll. Type Cultures) give methylthiol. Some of this is methylated to dimethyl sulphide (Challenger & Charlton, 1947). This fission may be reductive or hydrolytic or involve elimination of CH₃S— with a β-hydrogen atom. Alternatively, oxidative deamination may

give the α-keto acids CH₃SCH₂CH₂COCOOH (I) (obtained by Borek & Waelsch (1941) from methionine and liver or kidney slices) and



(II), which yields methylthiol with yeast and sucrose (Garreau, 1944). (I) gives methylthiol with hot acid or alkali (Waelsch & Borek, 1939), but we

find that (II) (Parrod, 1947) is stable under such conditions α -Keto- β -methylthiolpropionic acid (II) yields methylthiol and dimethyl sulphide (giving $(\text{CH}_3\text{S})_2\text{Hg}$ and $2(\text{CH}_3)_2\text{S} \cdot 3\text{HgCl}_2$) in glucose Czapek Dox cultures (pH=6.8) of *S. brevicaulis* α Keto γ methylthiolbutyric acid (I) yields methylthiol in bread cultures These keto acids may of course be reaminated to methionine and *S*-methylcysteine prior to fission β Methylthiolpropionic acid, $\text{CH}_3\text{SCH}_2\text{CH}_2\text{COOH}$ (IIA), also gives CH_3SH and $(\text{CH}_3)_2\text{S}$ in bread cultures of *S. brevicaulis* and CH_3SH in cultures on glucose-Czapek Dox medium No such decomposition occurs with



in bread cultures (Challenger & Charlton, 1947) Here elimination of $\text{C}_2\text{H}_5\text{S}$ — with a β hydrogen atom is impossible Control aspiration experiments with (II) and (IIA) in sterile bread gave no volatile sulphur compound (I) gave no CH_3SH or $(\text{CH}_3)_2\text{S}$, but a slight deposit in the HgCl_2 seemed to be due to presence of some CH_3SCH_3 This is under investigation

dl-Methionine methiodide gives $(\text{CH}_3)_2\text{S}$ but no CH_3SH in bread cultures of *S. brevicaulis* (Challenger & Charlton, 1947) Presumably therefore the C—S— fission of methionine does not proceed

through a sulphonium salt With *Penicillium notatum* on bread and on glucose Czapek Dox medium yields of 14 and 29% of dimethyl sulphide, but no methylthiol, are obtained Challenger & Simpson (1947) isolated the thetine derivative $^+ -$ $(\text{CH}_3)_2\text{S}(\text{Cl}) \cdot \text{CH}_2\text{CH}_2\text{COOH}$ (III) from the marine alga *Polysiphonia fastigiata* which, no doubt, provides the dimethyl sulphide which the alga evolves in air (Haas, 1935) Owing to the biological importance of sulphonium derivatives (du Vigneaud, 1942-3, Maw & du Vigneaud, 1948, Dubnoff & Borsook, 1948) and sulfoxides (Stoll & Seebeck, 1948, Reichstein, 1936) the behaviour of such compounds in cultures of *Scopulariopsis brevicaulis* and *Penicillium notatum* has been studied The thetine (III) is hardly attacked by *Scopulariopsis brevicaulis* but more readily by *Penicillium notatum*, the isomeric α derivative (IV) gives no dimethyl sulphide with either mould

Dimethylthetene bromide (V) gives no volatile sulphur compound, whereas diethylthetene bromide (VI) readily gives diethyl sulphide with *Scopulariopsis brevicaulis* None of the thetine salts (III)-(VI) shows any appreciable formation of dialkyl sulphide with 2N NaOH at ordinary temperature except (III)

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A Method of Quantitative Chromatographic Separation of Amino-acids By W S REICH (introduced by F CHALLENGER) (Department of Organic Chemistry, The University, Leeds 2)

A number of methods have recently been developed for the identification and the separation of amino-acids Tiselius (1940) introduced the method of chromatographic adsorption on active carbon To avoid the difficulties of chromatography of colourless substances, Karrer, Keller & Szönyi (1943) applied to amino-acids the method first introduced for the separation of sugars by Reich (1939), using the coloured *N* *p*-phenylazobenzoyl derivatives (or shorter azoyl derivatives) Gordon, Martin & Synge (1943) introduced the method of partition chromatography, based on differences in solubility of acetylamino acids in two immiscible solvents Sanger (1945) applied partition chromatography to the separation of the coloured *N*-2,4-dinitrophenyl derivatives of amino acids, first prepared by Abderhalden & Blumberg (1910)

Karrer's method has the advantage of being based on the adsorption properties which are more selective than the differences in solubility, and of using coloured derivatives, so that the separation may be controlled by following visually the development of the chromatogram However, the instability to acid hydrolysis of amino acid esters in general, and of the *N*-azoyl esters in particular, constitutes a serious obstacle to their employment in the study of many problems of protein constitution

It is known that in contrast to the *N* carboxyl esters, the *N* sulphonyl esters are stable to acid hydrolysis, and that the amino compounds can be recovered from such esters Having prepared *N*-azobenzene *p* sulphonyl derivatives (briefly to be called sulphazoyl derivatives) of some amino-

acid methyl esters, I find that they can be selectively adsorbed on suitably prepared alumina

The sulphazoyl derivatives were prepared by reaction of azobenzene *p* sulphonyl chloride with amino acid methyl esters in pyridine

The alumina for adsorption was prepared by treating aluminium oxide with a 10% solution of acetic acid in methanol

A mixture of 41 mg of sulphazoyl glycine methyl ester (m.p. 148–9°) and 44 mg of sulphazoyl DL alanine methyl ester (m.p. 127–8°) dissolved in 9 c.c. of chloroform, was passed through a column formed by a slurry of alumina (120 g), and a 0.1% methanol solution in benzene (250 c.c.) in a tube 22 × 400 mm. When all the solution of the sulphazoyl esters had been added to the column the

chromatogram was developed with 250 c.c. of the methanol/benzene solution. Each band was finally removed, and the adsorbed compounds were eluted with acetone. After evaporation of the acetone the melting points showed that the two esters had been separated. The appearance of the final chromatogram, and the weights of the recovered compounds were as follows

15 mm colourless	} 37 mg SA glycine methyl ester, m.p. 148–149°
60 mm deep orange	
10 mm colourless	
40 mm deep orange	} 39 mg SA DL alanine methyl ester, m.p. 129°
20 mm light orange	
50 mm deep orange	

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Amino-acid Decarboxylases of Lactobacilli By A. W. RODWELL (From the M.R.C. Unit for Chemical Microbiology, University of Cambridge)

Amino acid decarboxylases have not so far been recorded in lactobacilli. Six such enzymes have been described in other organisms, and, of these, the enzymes specific for lysine, tyrosine, arginine, ornithine and glutamic acid have been shown to require pyridoxal phosphate as co-enzyme (Gale, 1946). The histidine decarboxylase of *Cl. welchii* has not been resolved or otherwise demonstrated to require pyridoxal phosphate (Epps, 1945).

A number of strains of the genus *Lactobacillus* producing large amounts of histamine in culture were isolated from horse stomach contents. Washed suspensions of these organisms actively decarboxylate histidine, lysine and ornithine.

The histidine decarboxylase may be extracted from acetone dried cells by autolysis at pH 5. Attempts to resolve the histidine decarboxylase into specific apo enzyme and prosthetic groups by dialysis at 37° at pH values ranging from 2 to 9 were unsuccessful.

Pyridoxin is an essential growth factor for these

organisms, and the effect of pyridoxin deficiency on the formation of the complete enzymes was investigated. The lysine and ornithine decarboxylase activity of cells grown in pyridoxin-deficient medium was almost abolished, whereas histidine decarboxylase activity was found to be slightly greater in pyridoxin deficient cells.

The concentration of pyridoxin in the medium necessary for maximal ornithine and lysine decarboxylase activity is more than 1000 times greater than that required for maximal growth. The formation of the apo enzyme of the ornithine decarboxylase is not greatly affected by moderate pyridoxin deficiency, and the activity of washed cells grown under these conditions was found to increase (after a latent period) when the suspensions were tested in the presence of relatively high concentrations of pyridoxal. The duration of the latent period varied with the concentration of pyridoxal, and on the pH.

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The Effect of Anterior Pituitary Extract on the Metabolism of Isolated Rat Diaphragm By J H OTTAWAY and R H SMITH (introduced by L M KERLY) (*Department of Biochemistry, University College, London*)

Studies on the effects of anterior pituitary extracts (APE) *in vitro* on animal metabolism have been largely confined to experiments with isolated enzyme systems (Colowick, Cori & Slein, 1947), and attempts to show an effect of extracts added *in vitro* to intact tissues have given inconclusive results (Riesser, 1947). Our experiments have shown that while APE *per se* does not exert a marked influence on the metabolism of isolated rat diaphragm, it abolishes the increased uptake of glucose from a liquid medium containing insulin (Gemmell & Hamman, 1941, Stadie & Zapp, 1947).

For this purpose a half diaphragm, before being incubated in a phosphate medium containing glucose, was incubated for a half hour period with APE at 37°, a control half was incubated under similar conditions with 0.9% saline. The effect found was not an unspecific effect of protein, since incubation with extracts of muscle protein did not abolish the increased glucose uptake in the presence of insulin.

Under the usual experimental conditions (cf Gemmell & Hamman, 1941) insulin promotes an increase in glycogen synthesis, but this increase is not found in normal rat diaphragm which has been incubated either with saline or with APE. At the end of the half-hour period, however, the glycogen content of diaphragm incubated with APE is significantly lower than that incubated with saline (cf Nelson, 1944). Moreover, the APE-incubated diaphragm shows a lower glycogen content after incubation with high (4, 5 mg/ml) concentrations of glucose, but not with lower concentrations.

In diaphragm from adrenalectomized rats an increased synthesis of glycogen with insulin does occur after preliminary incubation with saline. No increase is shown after incubation with APE. The glucose uptake of diaphragm from adrenalectomized rats is at a generally high level (Krahl & Cori, 1947), and the increased uptake in the presence of insulin is still abolished by APE incubation.

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The Core of the Insulin Molecule By J A V BUTLER, D M P PHILLIPS and JOAN M L STEPHEN (*Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, W 1*)

Digestion of insulin with chymotrypsin results in the formation of a number of peptides of molecular weight *c* 800, and a residual core of molecular weight *c* 5000 (Butler, Dodds, Phillips & Stephen, 1948). These cores have been shown by electrophoresis and diffusion to be substantially homogeneous, the dimethylphenyl derivative runs on a paper chromatogram as one main spot, and a trace of a second which is possibly dimethylphenol. The size of the core, however, apparently depends on the concentration of enzyme used, as further digestion can be effected by treatment with more chymotrypsin.

After oxidation by the method of Toennies & Homiller (1942), as used by Sanger (1947) for insulin, the electrophoretic pattern of the core shows the presence of three or four constituents. These are also indicated by paper chromatography. Oxidation of the DNP core yields two fractions, one soluble and the other insoluble in dilute formic acid. The acid-insoluble portion can be dissolved in dilute am-

monia, and after neutralization with acetic acid is separable on a starch water column into three bands, similar to those obtained from the soluble portion.

DNP cores have also been examined for their terminal amino groups by the method of Sanger (1945). Terminal glycine, valine and histidine have been identified in three different cores, and a terminal ϵ amino group of lysine in two out of three. Ratios vary slightly from core to core, suggesting different degrees of digestion.

The distribution of aromatic amino acids between the precipitable core and the peptides in the filtrate has also been investigated. Phenylalanine determined by the Hess & Sullivan method (1944) is present in hydrolysates of the core and filtrate in the proportion of 1 to 4. Tyrosine determined directly by the Folin Ciocalteu reagent (1927) is distributed as three or four residues in the core, and five or six in the filtrate. These results support the

hypothesis that the chymotrypsin has acted on the peptide bonds involving the carboxyl group of an aromatic amino acid and the amino group

of another amino acid, in accordance with the specificity assigned to it by Bergmann & Fruton (1941)

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Diffusion of Hydrogen Ions through Isolated Gastric Mucosa By C TERNER (*Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, The University, Sheffield*)

It is generally believed that the HCl secreted by the oxyntic cell is isotonic with the blood, and that the variations in the acidity found in the gastric juice are due to subsequent dilution and neutralization by other secretions (see Babkin, 1944). Teorell (1933) rejected this view and suggested that back diffusion of H^+ ions through the gastric mucosa is the main cause of the variations.

To test this, manometric experiments were carried out to study the diffusion of H^+ ions through isolated gastric mucosa of frogs and toads in an apparatus described by Davies & Turner (1948). In thirty five experiments solutions of HCl + NaCl (total concentration 0.12 M) were placed in contact with the secretory sides of 0.5 cm² sheets of isolated mucosae which were incubated in bicarbonate saline at 25.0°. With non secreting mucosae an output of CO₂ in addition to the normal respiratory exchanges was observed which decreased exponentially with time in agreement with Teorell's findings. Teorell's (1947) equation $H = H_0 e^{-ct/p}$ was shown to apply (H_0 = initial acidity (mm), H = acidity at time t (min), p = volume (ml) of the added HCl solution, c = 'permeability coefficient'). In nine aerobic experiments with 0.1 N HCl, c for 0.5 cm² sheets of isolated mucosa of average thickness 0.3 mm was from 3.2×10^{-4} to 13.0×10^{-4} ml min⁻¹ (average 9.0×10^{-4} , standard deviation 2.7×10^{-4}). Similarly, in seven experiments with 0.05, 0.02 and 0.01 N HCl, c was from 2.2×10^{-4} to 11.0×10^{-4} ml min⁻¹ (average 7.0×10^{-4} , standard deviation 2.9×10^{-4}). There was, therefore, no significant difference in the

values of c obtained with these concentrations of HCl. The average diffusion constant for these mucosae calculated from the average value of c was 0.08×10^{-5} cm² sec⁻¹ (The diffusion coefficient of 0.1 N HCl is 2.5×10^{-5} cm² sec⁻¹ (Hoerber, 1945)).

Electrometric titrations of the HCl solutions after the incubation showed a reduction of acidity equivalent to the amount of CO₂ which had been evolved from the bicarbonate saline in excess of the respiratory exchanges. Similar results were obtained with anaerobic mucosa, dead pig bladder and Cellophane.

In aerobic mucosa in contact with 0.02 or 0.05 N-HCl the output of CO₂ stopped after stimulation by histamine, and the subsequent uptake of CO₂ indicated that HCl was being secreted (see Davies, 1948). Addition of 2 ml of 0.15 N HCl to 1 ml of 0.12 M NaCl already in contact with acid-secreting mucosa resulted in a decrease of the CO₂ uptake followed later by an output of CO₂, although fluid secretion continued at a slower rate.

In experiments with tied bags of gastric mucosa, using the technique of Davies (1948), back diffusion of secreted HCl was observed when secretion had stopped, but the rates of diffusion were only about one fifth of those observed in the experiments described above.

These results show that diffusion of H^+ ions can occur through living isolated gastric mucosa. The quantitative significance of diffusion in normal mucosa is, however, still uncertain, since the rates of diffusion are influenced by experimental conditions.

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Chemical Energy Relations in Gastric Mucosa By E EVA CRANE and R E DAVIES (*Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, The University, Sheffield*)

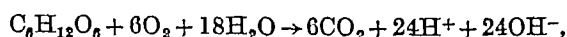
In order to study the relation between the energy liberated by metabolism and the energy utilized during functional activity, measurements were made of the average increases in the Q_{O_2} and the Q_{HCl} ($\mu\text{l}/\text{mg}$ dry wt/hr) following histamine stimulation in each of thirty-three isolated frog gastric mucosae in phosphate saline gassed with 100% O_2 (for technique see Davies, 1948)

Under conditions obtaining in acid secreting frog oxyntic cells, the minimum free energy needed to produce the H^+ and Cl^- ions together with the water of the secretion has been calculated to be about 1.0×10^4 cal/g mol HCl

The free energy from glucose oxidation which is available for secretion is unknown but some estimates can be made. Two possibilities are discussed (A) If all the free energy of the overall reactions between glucose and O_2 is available, then for the secretory units $Q_{HCl}/|Q_{O_2}|$ cannot be greater than 11.5 (B) If the free energy of the reactions between glucose and ferricytochrome *c* only is available (ferri/ferro = $\frac{1}{10}$), then energy is dissipated in the later stages of the oxidations, and for the secretory units $Q_{HCl}/|Q_{O_2}|$ cannot be greater than 6.0

An electron-cycle mechanism (Crane, Davies & Longmuir, 1948) has been suggested for the utilization of this metabolic energy (perhaps as high-energy phosphate bonds) in HCl secretion. A possible supplementary process for the production of H^+ and OH^- ions by oxyntic cells (suggested independently by Robertson (1948)) is the reaction of O_2 with substrate H atoms, via cytochrome oxidase and the cytochromes, to form H^+ and OH^-

instead of H_2O . The overall reaction for glucose oxidation is then



and by this process alone (C) $Q_{HCl}/|Q_{O_2}| = 4$ for the secretory units. If the increase in metabolism after histamine provides the power used for acid secretion, then the ratio

mean Q_{HCl} for the period of increased respiration after histamine
 mean increase in Q_{O_2} for this period

is the same for the whole mucosa as for the secretory units. The results presented show that, within experimental error, this ratio < 11.5 for all of thirty-three mucosae, < 6.0 for only half of the mucosae and < 4 for only one-third of them.

The thermodynamic efficiency of HCl production appears to be remarkably high, on the basis of (A) it is 50% or more for about two-thirds of the mucosae used, either (B) or (C) alone gives many results of over 100%. It may be that part of the energy or some of the H^+ ions from the unstimulated respiration can be utilized for acid secretion, in which case (B) or even (C) alone may not be excluded, since the mean Q_{HCl} of the mucosa for the whole experiment was never greater than the mean Q_{O_2} . This result is expected when phosphate saline is used (Davies & Longmuir, 1948).

Maximally secreting mammalian oxyntic cells can have Q_{HCl} values greater than 1000. For 100% efficiency a Q_{O_2} of -87 is required assuming (A), -170 assuming (B) and -250 assuming (C) alone. These high values, which must be exceeded if the cells are less than 100% efficient, show that they are among the most active mammalian cells known.

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Electric Energy Relations in Gastric Mucosa By E EVA CRANE and R E DAVIES (*Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, The University, Sheffield*)

When isolated frog gastric mucosa commences to secrete HCl, its capacity to do external electrical work decreases (Crane, Davies & Longmuir, 1948a). Moreover, electrical energy supplied by an external source of power can apparently be utilized by acid-secreting mucosa (Crane, Davies & Longmuir, 1948b). An attempt has therefore been made to correlate the electrical and biochemical energy relations of HCl secretion.

The table gives data for an average isolated frog gastric mucosa at 25.0°. The latent electric power is the maximum electric power the mucosa might be expected to expend (see Crane *et al.* 1948a). Since 1 cal/sec = 4.2 W, for a non-secreting mucosa this represents 3.6% (A) or 6.3% (B) of the metabolic power of the whole mucosa (see Crane & Davies, 1948). During HCl secretion the latent electric power is lower by 0.30 $\mu\text{W}/\text{mg}$ dry wt. Since the

secretion of 1 g mol HCl requires 1.0×10^4 cal, a Q_{HCl} of 2.0 requires a power of $1.0 \mu W$ /mg dry wt, i.e. $0.7 \mu W$ /mg dry wt more than the maximum which might be available from the latent electric power. Now the average increase in $|Q_{O_2}|$ of 0.3

Table 1 *Physical and metabolic data for an average isolated frog gastric mucosa at 25°C, of thickness 0.25 mm and dry wt in 1 cm² of holder, 9.5 mg*

Variable	Value before HCl secretion	Value during HCl secretion	Change
Q_{O_2} (μl /mg dry wt/hr)	-2.1	-2.4	-0.3
Q_{HCl} (μl /mg dry wt/hr)	0	2.0	2.0
Potential difference (mV)	-30	-20	10
Resistance of mucosa in 1 cm ² of holder (Ω -cm ²)	210	280	70
Latent electric power, i.e. p.d./resistance, for 1 mg dry wt (μW /mg dry wt)	0.45	0.15	-0.30

on secretion would provide 1.8 (A) or 0.93 (B) μW /mg dry wt. This would imply an efficiency of at least 39% (A) or 75% (B) in the utilization of energy from the increase in the rate of respiration.

When an enhancing current is passed through an isolated frog gastric mucosa secreting HCl,

the Q_{HCl} increases (Crane *et al.* 1948b). With 0.4 ma/cm² the average increase in Q_{HCl} was 1.0, this requires an additional power of $0.5 \mu W$ /mg dry wt. The power taken from the external source cannot be calculated until the mechanism of energy transfer is more fully understood, but some estimates can be made. According to the electron cycle mechanism of HCl production, an electron is transferred across a Fe^{++} , Fe^{+++} system for each H^+ and Cl^- ion secreted (Crane *et al.* 1948a). This process could be expedited by the potential difference maintained across the secretory units by an enhancing current.

For a given mucosa the Q_{HCl} is proportional to the rate of transport of H^+ ions (and of electrons). Assuming that this rate is proportional to the total potential difference across the units, the calculated increase in Q_{HCl} agrees with the experimentally observed value. The power required to maintain this enhanced potential difference is at least (1) that needed to overcome the resistance of the mucosa (Joule effect), i.e. $4.7 \mu W$ /mg dry wt for the average mucosa secreting HCl, and (2) that needed to overcome the e.m.f. of the mucosa, i.e. $0.84 \mu W$ /mg dry wt. The average overall efficiency of the process would thus be about 10%.

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The Thyroid Gland and Carotene and Vitamin A Metabolism By T. W. GOODWIN (Department of Biochemistry, The University, Liverpool)

The work of Drill & Truant (1947), Canadell & Valdecasas (1947), and Johnson & Baumann (1947) indicates that either thyroidectomy or treatment with thiouracil impairs the ability of rats to convert carotene into vitamin A. Johnson & Baumann also showed that desiccated thyroid counteracts the effect of thiouracil, and that hyperthyroid animals convert carotene into vitamin A more efficiently than do normals. The thyroid hormone or the drug may act directly on the enzyme system concerned in the carotene \rightarrow vitamin A transformation, or indirectly by affecting the stability of carotene in the digestive tract or by controlling the efficiency of absorption of carotene. An attempt is being made to resolve these uncertainties.

Absorbed carotene is converted into vitamin A in the gut wall (see, for example, Glover, Goodwin & Morton, 1948) and in animals such as goats and rabbits this process is apparently so efficient that carotene never appears in the blood (Goodwin &

Gregory, 1948). Inhibition of the enzyme system not accompanied by reduced absorption or reduced

Table 1 *Vitamin A blood levels of rabbits dosed with either thiouracil or desiccated thyroid*

Animal no.	Dose	Blood vitamin A levels (i.u./100 ml plasma) Days after beginning of administration of drug				
		0	7	14	21	28
3	Control	138	154	148	159	142
4	{ 500 mg thiouracil per day for 18 days }	171	166	172	149	143
6		—	169	170	149	165
7	200 mg desiccated thyroid per day for 18 days	127	148	126	123	121

intestinal stability of carotene should result in the appearance of carotene in the blood. Adult rabbits maintained on a carotene rich diet, were treated for

3 weeks with thiouracil and their blood examined periodically for carotene, on no occasion was carotene found. As thiouracil has no effect on the stability of carotene *in vitro* (Cama & Goodwin, 1948), the possibility exists that the primary action of thiouracil is on the absorption of carotene. Experiments to test this are in hand.

The plasma vitamin A levels of the animals just considered and of similar animals treated with desiccated thyroid were also followed, no significant variations were recorded in any group (Table 1),

and no intermediate metabolites (e.g. retinene) were detected.

Whatever the action of the thyroid hormone on vitamin A metabolism *per se* it does not appear to control the system maintaining the equilibrium between plasma and liver vitamin A levels (Glover, Goodwin & Morton, 1947). This fact suggests that the diagnostic value of vitamin A plasma levels in cases of suspected thyroid imbalance is of little value, it can also account for the conflicting clinical reports (see e.g. Drill, 1943).

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The Role of Thyroglobulin and Iodinated Casein in the *in vitro* Conversion of Carotene into Vitamin A

By H. R. CAMA and T. W. GOODWIN (Department of Biochemistry, The University, Liverpool)

Kaplansky & Balaba (1946) have reported that carotene is converted into vitamin A by incubating colloidal solutions of carotene with aqueous solutions of either thyroglobulin or iodinated casein, boiled solutions of the proteins are ineffective. The implications of such a reaction in the study of vitamin A metabolism are so important that an investigation of the Russians' claim has been undertaken. Their experimental details have been followed as closely as possible but they are somewhat scanty. No conversion was ever demonstrated but colloidal solutions of β carotene when incubated alone or with boiled or unboiled thyroglobulin or iodinated casein undergo considerable stereoisomerization. This isomerization alters the absorption spectrum of β -carotene and results in the formation of a 'cis' peak at c. 335 m μ (Zechmeister, 1944). This band could conceivably be mistaken for the vitamin A band (328 m μ), and further evidence, such as that provided by the SbCl_3 colour test, is necessary, even then care must be taken to avoid mistaking the vitamin A 'blue' for the β carotene 'blue'.

Kaplansky & Balaba state that both ultra-violet and SbCl_3 tests were carried out, but no indication of the instruments used are given.

In the present investigation the correction procedure of Morton & Stubbs (1946) was applied to the ultra violet absorption spectra of incubated solutions measured on a Beckman photoelectric spectrophotometer, none of the spectra was 'correctable' for vitamin A. Using the Hilger-Nutting spectrophotometer, by which the SbCl_3 colours of vitamin A and β carotene can be easily distinguished, the absence of vitamin A was confirmed.

After such negative results the investigation was expanded in a series of experiments carried out under varying pH, incubation temperatures and times, freshly minced bullocks' thyroids were also used. Vitamin A formation was never demonstrated.

The stability of incubated colloidal solutions of β carotene was unaltered by the addition of thiouracil.

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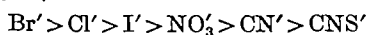
Some Properties of the Glutaminase of *Clostridium welchii* By D E HUGHES and D H WILLIAMSON
(Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, The University, Sheffield 10)

As previously shown, cetyltrimethyl ammonium bromide (cetavlon) accelerates the decarboxylation of glutamic acid and the deamination of glutamine by suspensions and cell free extracts of *Cl. welchii* (Krebs, 1948, Hughes, 1948). To study the cause of this acceleration the properties of the glutaminase have been investigated.

The activity of the glutaminase was determined by measuring the NH_3 formation in a mixture containing 0.01 M-glutamine, 0.1 M acetate buffer, pH 5.0, 0.025 M KCl and the enzyme material. The rate of glutamine hydrolysis is expressed as Q_{NH_3} ($\mu\text{l NH}_3/\text{mg dry wt/hr}$).

Through the courtesy of Dr B C J G Knight centrifuged cells of *Cl. welchii* (Str 107) CN 1490 from 500 l of medium grown for the preparation of α toxin were available. These were dried over P_2O_5 and extracted with 6 vol of borate KCl buffer at pH 8.5. The supernatant, after extraction, contained 80% of the original activity. This was further fractionated by precipitation at pH 7.0, 4.8 and 4.1. The precipitate obtained at pH 4.1 contained 12% of the dry weight and 50% of the glutaminase activity of the original cells. Q_{NH_3} was 1300 compared with 350 for the starting material. The purified enzyme was soluble in phosphate buffer at pH 8.0–8.5 but not at a lower pH.

Dialyzing or washing the precipitated enzyme with water, resulted in the almost complete loss of activity. This could be fully restored by NaCl or KCl (2.5×10^{-2} M). Other anions replaced Cl^- in the following order of effectiveness:



The glutaminase of the cells used for the preparation of the purified enzyme was not accelerated by

cetavlon. Apart from belonging to a different strain, the cells differed from those which had been previously used by having been harvested after 5 hr, compared with 16 hr incubation in the previous work. This suggested that the age of the culture determined the cetavlon effect. The table shows that this is the case. It also shows that although there was no further growth after $5\frac{1}{2}$ hr, the glutaminase activity continued to increase. About two thirds of the glutaminase activity of the cells harvested at $13\frac{1}{2}$ hr was formed after growth had stopped, and it thus appears that cetavlon acts only on the enzyme formed after cessation of

*Age of culture and glutaminase activity of
Cl. welchii (Str 107) CN 1490*

(Cells grown at 37° on casein meat glucose medium, washed once with saline)

Time (hr)	Dry wt cells (mg/ml)	pH of medium	Q_{NH_3}	
			Without cetavlon	Cetavlon (0.0025 M)
0	—	6.8	—	—
$2\frac{1}{2}$	0.13	6.3	36	36
4	0.37	5.1	85	85
$5\frac{1}{2}$	0.43	4.8	112	154
7	0.43	4.7	156	212
$13\frac{1}{2}$	0.40	4.7	256	425

growth. A cetavlon effect could be induced in washed young cells by suspending them in buffers at pH 4.0 or 5.0 for 3 hr at 40° . This treatment abolished 80% of the original activity, and cetavlon restored this. The activity of the purified enzyme was not increased, but inhibited by cetavlon.

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The Effect of Fats and Fatty Acids on Lactobacilli Used in Microbiological Assays By F W NORRIS and K J LYNES (Department of Industrial Fermentation, University of Birmingham)

The present investigation arose from studies of biotin content of ungerminated and germinated cereals. By minor modifications in the method of assay of biotin by Barton-Wright (1946), improved response curves using *Lactobacillus arabinosus* 17/5 were obtained. The casein hydrolysate used in the medium was standardized to contain 10% N (Kjeldahl). Increased buffer action in the medium

was effected by raising the concentration of sodium acetate from 0.6 to 0.9%.

In the assay of cereal products it became evident that the most serious cause of trouble was the presence of fats in the original material or of fatty acids in the prepared extracts, as reported and discussed by a number of workers. In general, the presence of these substances inhibits the activity of

the assay organisms and causes a 'downward drift' in assay results at increasing vitamin dosage levels. The problem is complex and may involve both chemical and physical effects.

In the biotin assay, using oils prepared from cereals, other vegetable oils, and an animal oil such as cod liver oil, it was found that, after initial stimulation at the basal vitamin level, there was a change over to inhibition at higher vitamin levels. The degree of inhibition appeared to increase with increasing iodine number of the oils. With the fatty acids themselves, saturated acids—palmitic and stearic—behaved similarly to the oils, whereas unsaturated acids—oleic and linoleic—caused considerable stimulation, irrespective of vitamin level.

It is difficult to reconcile these results with explanations offered hitherto. Since unsaturated acids play an important part it is probable that labile oxidation-reduction systems are involved. It seems unlikely, however, that the influence of redox potentials on bacterial growth, propounded by

Knight & Fildes (1930), involves stimulation or inhibition depending on vitamin concentration.

Again, our results do not lend themselves to complete support of the suggestions of Kodicek & Worden (1945). A large number of experiments on *L. helveticus* were carried out on similar lines to those proposed by Kodicek & Worden. Although confirmation of some of their results was obtained, it was found that, in an 'unextracted' medium, oleic acid did tend to inhibit growth, although some 'protection' against this inhibition was afforded by fatty substances in the medium. It was also found that if the organisms were first allowed to attain active growth in normal media, subsequent addition of unsaturated acids produced but slight effect. It is thus difficult to accept the suggestion of Kodicek & Worden that the action is physico-chemical and due to the formation of an inhibitory mono layer of fatty acids round the bacteria. It is thought that an alternative suggestion of these workers is more probable, namely, that these acids have a direct chemical action on the metabolism of the bacteria.

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The Effect of Phosphate on Periodate Oxidation of some Carbohydrates By D. J. BELL, ANNE PALMER and A. T. JOHNS (*Biochemical Laboratory, University of Cambridge*)

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The Influence of some Synthetic Oestrogens and Related Substances upon the Succinoxidase System of Rat Liver

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Practically nothing is known concerning the manner in which oestrogens, either natural or synthetic, bring about their characteristic effects *in vivo*, and the difficulty of correlating oestrogenic activity with chemical structure is notorious. Particular interest, therefore, attaches to the observations of McShan & Meyer (1946) on an apparently specific inhibition of the succinoxidase enzyme complex in rat liver and pituitary tissues, produced by stilboestrol (4,4'-di-hydroxy α,β -diethylstilbene), hexoestrol and dienestrol in low concentrations. These workers indicated that the inhibition was exerted not upon the succinic dehydrogenase, but upon the cytochrome oxidase component of the system, being thereby distinguished from inhibitions of the type produced by malonate, from which it also differed in respect of the markedly lower concentrations of oestrogen necessary to inhibit to a given degree. When one or both of the 4,4'-phenolic hydroxyl groups were replaced by carboxymethoxy groups it was reported that the inhibitory power of the resulting compounds towards succinoxidase and the oestrogenic potency declined in parallel. These findings, as was pointed out by the authors, suggest that the oestrogenic effect of such compounds is functionally associated with their action upon the succinoxidase system. This possible elucidation of the mode of action of oestrogens acquires an enhanced significance in the light of the relationship existing in some cases between oestrogenicity and carcinogenicity. If the elicitation of an oestrous or neoplastic response in the living organism could be shown to be consistently associated with the ability of the agent to interfere with a specific enzymic reaction, a fundamental contribution to our understanding of these processes would have been made. We have, therefore, amplified the observations of McShan & Meyer (1946) by investigating the action of a wider range of oestrogens and of substances of related chemical structure whose oestrogenic potency is slighter or in some instances negligible. The hypothesis that oestrogenicity is correlated with the property of inhibiting some component of the succinoxidase system is not supported by our results. Since this work was completed a further communication by McShan, Meyer & Trwax (1947) has appeared in which the concept is likewise abandoned. Some of our experiments,

however, throw interesting light upon the question of the constitution of the succinoxidase system. Evidence has been previously adduced that succinate, activated by its dehydrogenase, is not, as was once thought, capable of reacting directly with the cytochrome c cytochrome oxidase complex, nor of reducing cytochrome c without the intervention of other intermediates, and the findings reported here support this view.

EXPERIMENTAL

In general the technique of McShan & Meyer (1946) was followed closely. As a source of the succinoxidase system and its components, homogenized rat liver was used. Adult males from the laboratory stock were killed by decapitation and the appropriate amounts of tissue excised, weighed and homogenized immediately in ice cold glass distilled water by the method of Potter & Elvehjem (1936). The activity of the homogenates was unaffected by storage for several hours at 0°, and it was found advantageous as a routine to allow them to stand for 2 hr. in the refrigerator, and then to decant the supernatant suspension from the slight sediment of coarse undissolved cell debris. This yielded very uniform dispersions which could be accurately pipetted and gave highly reproducible results, replicates commonly agreeing to within 2 or 3%. Preparations which had been kept for 24 hr. or more at 0° usually retained over 80% of their succinoxidase activity, but in view of possible differential deterioration of the constituents of the enzyme system, results obtained with such homogenates are not included. The final homogenate was customarily used in a concentration of 5% (in terms of fresh tissue) for succinoxidase or succinic dehydrogenase assay, and 1 or 0.5% for cytochrome oxidase determination. The equivalent dry weights of tissue were determined by drying samples of homogenate to constant weight at 105–110°.

Cytochrome c was prepared from heart muscle of freshly slaughtered horses by the method of Keilin & Hartree (1937), the only modifications being that glass distilled water was used instead of dilute NaCl for the final dialysis, and that the product was stored in the refrigerator in aqueous solution ($10^{-3}M$) without chloroform or other preservative. Standardization was carried out spectrophotometrically with the Hilger Nutting instrument, and the preparations showed no evidence of deterioration over a period of several months.

Sodium succinate was prepared by accurate neutralization of a concentrated aqueous solution of pure succinic acid with NaOH, followed by precipitation and washing with ethanol, filtration and drying.

The substances tested for inhibitory activity are listed in Table 1. We are indebted to Mr W. Lawson of this laboratory for carrying out the synthesis of the majority and for generously presenting us with specimens. The oestrogenic potency was assayed in this laboratory by the usual routine technique, using rats (Wilder, Smith & Williams, 1947). The 'oestrogenically active dose' is the total weight of compound which, when administered in 5 or 6 injections over a 3 day period, results in 100% positive response. The method by which solutions of these compounds were obtained was substantially that outlined by McShan & Meyer (1946), they were dissolved in small quantities of 2N NaOH, which was then partly neutralized with HCl and subsequently diluted with distilled water, the quantities being adjusted so that the resulting solutions were 0.006M with respect to NaOH, 0.002M with respect to NaCl, and of the desired molarity, usually between 0.0005 and 0.002M, with respect to the substance under test. Sometimes poor solubility of the inhibitor necessitated stronger alkali, in all cases appropriate control solutions were made up with NaOH and HCl and added to the control manometric vessels in the experiments. Inconsistencies in preliminary results were traced to the circumstance that the oestrogens or inhibitors were occasionally partially, or wholly, precipitated from their alkaline solutions on being mixed with the rest of the contents of the reaction vessels, which were buffered at a lower pH, i.e. 7.4. Attempts to overcome this difficulty by such expedients as the use of caffeine as a solubilizer (Weil-Malherbe, 1946) were unsuccessful because purines in the concentrations necessary inhibited the succinoxidase system powerfully, the use of more alkaline buffers to prevent precipitation also severely impaired the enzymic activity. Solutions of some of the compounds were supersaturated, and often spontaneously deposited crystals within an hour or two, for this reason they were always made up immediately before use. It was found that the tendency to precipitate in the reaction mixtures was much reduced if the inhibitors were introduced into the Warburg flasks before any of the other constituents of the reaction mixture other than water, and this was made standard routine. In addition, 'pilot' mixtures were set up in which samples of the inhibitor solutions were added to mixtures which imitated the contents of the experimental vessels except that homogenate and cytochrome were omitted, it was then possible to be reasonably certain whether the substances were or were not remaining in solution.

Conventional Warburg manometric technique was employed. When homogenates were used, the procedures for assay of succinoxidase and cytochrome oxidase described by Schneider & Potter (1943) were followed in essentials. For succinoxidase, each vessel contained 0.2 ml. of enzyme preparation, 0.2 ml. of a solution 0.006M with respect to both AlCl_3 and CaCl_2 , 0.2 ml. of 0.75M Na succinate, 0.1 ml. of 10^{-3} M cytochrome *c*, 0.3 ml. of inhibitor or control solution, 1.0 ml. of phosphate buffer (0.1M, pH 7.4), and glass distilled water to make a total volume of 3.0 ml. In cytochrome oxidase investigations the succinate was replaced by 0.3 ml. of 0.114M Na ascorbate (freshly prepared by neutralization of ascorbic acid with NaOH), 1.0 ml. of 10^{-3} M cytochrome *c* was used instead of 0.1 ml., and the concentration of the homogenate was reduced from 5 to 0.5%. For the measurement of succinic dehydrogenase activity, cytochrome was omitted, and 0.5 ml. of

a 0.5% aqueous solution of brilliant cresyl blue (Weil-Malherbe, 1937; McShan & Meyer, 1946) was included, sometimes cyanide in 10^{-3} M concentration was present also. In all experiments with homogenates the gas phase was air.

Slices and strips of tissue were used in a phosphate Ringer solution, pH 7.4 (Dickens & Šimer, 1930), to which Na succinate (0.0375M) and dissolved oestrogen (10^{-4} M) or control solution were added. The centre wells contained 0.2 ml. of 10% NaOH, the total fluid volume was 2.2 ml., and the gas phase was O_2 . Flasks were shaken at 96 oscillations/min in a water bath at 37.5°. Spectroscopic observations were made either with a hand spectroscope or with a Hilger constant deviation wave length spectrometer.

RESULTS

Under the conditions stated the Q_{O_2} for succinate oxidation by the liver homogenates was consistently in the neighbourhood of 70 in the controls, rarely falling below 65 or exceeding 75. This is in fairly good accord with the figures of McShan & Meyer (1946) and other American workers, though it agrees poorly with recent values given by Hoch-Ligeti (1947) in this country, which on the average were of the order of half this magnitude. However, the discrepancy may be largely accounted for by the fact that we used 10 mg. of wet tissue in 3 ml., with the addition of calcium and aluminium ions, whereas Hoch-Ligeti (1947) used 40 mg. of tissue in the absence of Ca^{++} and Al^{+++} . For the oxidation of ascorbic acid by cytochrome oxidase the range of variation in Q_{O_2} was somewhat wider, but the values were always between 300 and 400.

When succinic dehydrogenase activity was measured in the presence of brilliant cresyl blue, with or without cyanide, but in the absence of added cytochrome *c*, the Q value was generally reduced to less than half of its normal level, i.e. to about 30. Some specimens of this dye seemed more injurious to the enzymes than others, their use was naturally avoided when possible. Methylene blue proved markedly less efficient as a carrier, and after preliminary trials was not employed in these experiments.

Inhibition of succinoxidase in liver homogenates

Table 1 contains the summarized results showing the overall inhibition of succinoxidase by the whole range of compounds tested, presented in the form of percentage inhibitions in relation to the control values, for three levels of concentration of inhibitor. These inhibitions have been calculated on the basis of the average Q_{O_2} over the first four 10 min. periods, the uptake was almost invariably nearly linear for at least twice that time. Several tests were made with each substance, flasks were set up in duplicate, and habitually gave agreement to within less than 5%, the mean value being taken. The figures in

Table 1 are taken from the protocols of single typical experiments, and are fully representative since discordant or anomalous results were not encountered, except on a few occasions when the inhibitor had precipitated from solution

almost negligible inhibitory power, while their effectiveness as oestrogens is considerable. The most convincing evidence is perhaps that furnished by bisdehydrodisynolic acid methyl ether and 2-(6'-methoxy-2' naphthyl)-1,1 dimethyl-*n*-valeric acid,

Table 1 *Structure, oestrogenic potency and inhibitory power towards succinoxidase of compounds tested*

(The compounds are grouped according to their activity on the basis: high oestrogenic activity = oestrogenic dose < 1 mg, high inhibitory power = $> 50\%$ inhibition at 2×10^{-4} M.)

Compound	Oestrogenic dose	Percentage inhibition at molar conc		
		5×10^{-5}	10^{-4}	2×10^{-4}
A. High oestrogenic and high inhibitory activity				
Stilboestrol (4 4'-dihydroxy- α,β -diethylstilbene)	<0.5 μg	60	87	100
Hexoestrol	0.2	51	83	100
Dienoestrol	<0.5	17	78	95
B. High oestrogenic and low inhibitory activity				
Sodium hexoestrol diphosphate	2 μg	1	—	3
Sodium hexoestrol monosulphate	10	7	12	20
Dipotassium hexoestrol disulphate	25	0	3	8
Dipotassium stilboestrol disulphate	25	9	10	—
4 4'-Dicarboxymethoxystilboestrol (sodium salt)	200	—	0	5
4 4'-Dicarboxymethoxyhexoestrol (sodium salt)	200	—	18	22
7-Methoxy-2-methyl-1-ethyl-1,2,3,4-tetrahydrophenanthrene-2-carboxylic acid (bisdehydrodisynolic acid methyl ether)	0.5	1	3	9
2-(6'-Methoxy-2'-naphthyl)-1,1-dimethyl- <i>n</i> -valeric acid	3	5	7	13
C. Low oestrogenic and high inhibitory activity				
isoHexoestrol	1 mg	27	57	99
4 4'-Dihydroxystilbene	10	65	90	97
1-(4'-Hydroxyphenyl)-2-phenylethane	>100	12	26	52
1,2-Di(4'-hydroxyphenyl)cyclohexane	>10	8	25	82
4-Hydroxybenzylideneacetophenone	100	22	42	60
Benzylidene-4-hydroxyacetophenone	>100	15	30	58
4- <i>tert</i> -Amylphenol	100	15	32	—
4 4'-Dihydroxytriphenylmethane	25	27	53	100
3-Phenyl-3-(4'-hydroxyphenyl)-2,4-dimethylpentane	2	50	92	—
2-Phenylphenol	>100	30	65	—
2-Phenyl-2-(4'-hydroxyphenyl)propane	50	30	55	98
D. Low oestrogenic and low inhibitory activity				
4-Hydroxystilbene	10 mg	9	26	45
1,2-Di(4'-hydroxyphenyl)ethane	100	7	11	23
4-Hydroxydiphenylmethane	>100	0	20	34
4 4'-Dihydroxydiphenylmethane	100	5	10	23
4 4'-Dihydroxydiphenyl	100	2	4	11
Epoxy-2,3-di(4'-hydroxyphenyl)butane	>10	1	3	9
1,2-Di(4'-hydroxyphenyl)cyclohex-1-ene	>10	8	20	40
3,4-Di(4'-hydroxyphenyl)hexane-1,6-diol	>10	0	1	11
4 4'-Dihydroxydibenzylideneacetone	>100	11	16	19
1-Naphthol	>100	7	—	25

Table 1 shows that there is no systematic correlation between oestrogenic and inhibitory powers. It is true that the potent oestrogens stilboestrol, hexoestrol and dienoestrol strongly inhibit succinoxidase, but racemic isohexoestrol, whose oestrogenic potency is about 1/5000 that of *meso*hexoestrol, exhibits only slightly less inhibitory capacity. The sodium and potassium salts of the sulphates, phosphates and carboxymethoxy derivatives (so called oxycetates or substituted glycolic acids) possess

which are among the most powerful synthetic oestrogens known, and yet exert virtually no inhibition upon the succinoxidase system. Compounds which exert powerful inhibition of succinoxidase, but possess low oestrogenic activity, include 4,4'-dihydroxystilbene, the two benzylideneacetophenones, 4-*tert*-amylphenol, 4,4'-dihydroxytriphenylmethane, 3-phenyl-3-(4'-hydroxyphenyl)-2,4-dimethylpentane, 2-phenyl-2-(4'-hydroxyphenyl)propane and 2-phenylphenol.

The effect of oestrogens on the metabolism of tissue slices and strips

There is no great effect of this group of substances on the respiratory rate of sliced or intact tissues. Fig 1 shows a slight stimulating effect of hexoestrol and isohexoestrol upon the respiration of liver slices in the absence of added succinate, when succinate is present as substrate, however, the effect is abolished. Inhibitory effects similar to those observed with homogenized tissue were not seen.

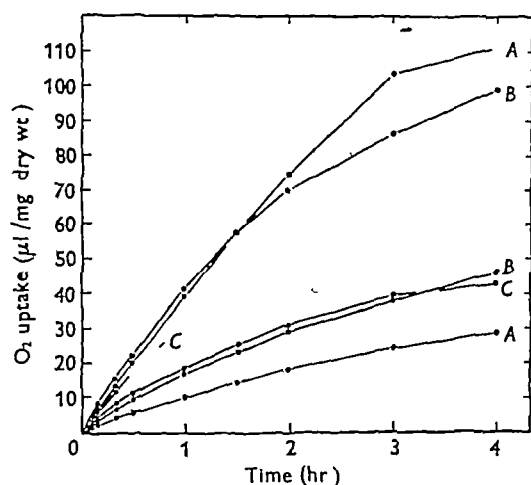


Fig 1 Effect of oestrogens on metabolism of rat-liver slices. Lower three curves, no succinate, upper three curves, succinate present. A, no oestrogen, B, hexoestrol 10^{-4} M, C, isohexoestrol 10^{-4} M. (Succinate + isohexoestrol record does not extend beyond 30 min. owing to experimental mishap.)

Since oestrogenic hormones *in vivo* have a pronounced effect on the uterus and vagina, it was of interest to examine the *in vitro* effect on these organs. The tissues were dissected from freshly killed ovariectomized rats in dioestrus, and were introduced into the Warburg flasks with no treatment beyond being opened up with scissors so that their entire surfaces were freely accessible to the liquid in the vessels. The Q_{O_2} was measured in the usual way, using succinate as added substrate, but no significant differences were detectable between the controls and the systems containing oestrogen (dienoestrol).

Effect of oestrogens on the metabolism of homogenates

These inhibitors might conceivably act by stimulating the oxidative formation from malate of oxaloacetate, which powerfully inhibits succinic dehydrogenase, and it has been shown by Swingle, Axelrod & Elvehjem (1942) that the probable function of Ca ions in accelerating the oxidation of succinate (whence their inclusion in the reaction mixtures employed in the work described in this paper) is that of assisting in the enzymic destruction of coenzyme 1, the latter being essential for the formation of oxaloacetate. Oxaloacetate may also be removed by transamination with glutamate, yielding α keto glutarate and aspartate, and Swingle *et al* (1942) found that glutamate reverses the inhibition of the succinoxidase system caused by oxaloacetate, and stimulates the succinoxidase system to an extent comparable with the effect of calcium. An experiment was carried out to test the effect of L-glutamate (Table 2), but no evidence was obtained that its presence affected in any way the inhibition produced by stilboestrol.

McShan & Meyer (1946) considered that the locus of the inhibition was the cytochrome oxidase component of the succinoxidase system, since they found that a concentration of oestrogen which inhibited the complete succinoxidase system to a pronounced degree had only a feeble effect upon succinic dehydrogenase, whereas cytochrome oxidase was markedly affected. Using 10^{-4} M stilboestrol, their figures show an average inhibition of 76% for succinoxidase, 52% for cytochrome oxidase, and 11% for succinic dehydrogenase. This evidence for attributing to stilboestrol an exclusive effect upon cytochrome oxidase is not entirely convincing, and our own values are even less so, indicating in general a distinctly higher figure, in the region of 30%, for the inhibition of the dehydrogenase.

In order to investigate this matter further, and especially to ascertain whether the mechanism of interference with the succinoxidase system by these inhibitors is the same irrespective of their oestrogenic potency, we selected from the compounds listed in Table 1 a few showing marked inhibitory power but feeble oestrogenicity, and studied their effects upon the three systems, succinoxidase, cytochrome oxidase and succinic dehydrogenase.

Table 2 Influence of glutamate on the inhibition of succinoxidase due to stilboestrol

(Ca^{++} and Al^{+++} omitted from reaction mixtures. Concentration of stilboestrol 10^{-4} M, of L-glutamate 5×10^{-2} M.)

	Control	Stilboestrol	Glutamate	Stilboestrol + glutamate
Q_{O_2}	63.3	18.5	63.9	17.0
Inhibition due to stilboestrol (%)	—	71	—	74

The following were chosen 2 phenylphenol, 4 4'-dihydroxystilbene, 1(4'-hydroxyphenyl) 2 phenylethane and 4 hydroxybenzylideneacetophenone. The first two in 10^{-4} M concentration and the second two in 2×10^{-4} M concentration inhibit succinoxidase in liver homogenates by 50–100%. Stilboestrol was also included as a representative of the class of strong inhibitors which are powerfully oestrogenic. The results summarized in Table 3 were all obtained with the same preparation of rat liver homogenate

may preponderate over that of the latter, and vice versa. But one compound, 4 4'-dihydroxystilbene, stands out as of peculiar interest in that, while second only to stilboestrol in its ability to inhibit the oxidation of succinate by the complete system, it has no effect upon succinic dehydrogenase and virtually none upon cytochrome oxidase. Further experiments showed that, even in a concentration which almost completely abolished the overall oxidation of succinate, the cytochrome oxidase and

Table 3 *Differential inhibition of components of the succinic oxidase system*

Inhibitor (M)	Percentage inhibition of enzyme system (calculated on first 40 min)		
	Complete succinoxidase system	Succinic dehydrogenase	Cytochrome oxidase
Stilboestrol (10^{-4})	86	33	57
4 4' Dihydroxystilbene (10^{-4})	78	0	3
2 Phenylphenol (10^{-4})	61	29	0
4-Hydroxybenzylideneacetophenone (2×10^{-4})	56	47	10
1(4' Hydroxyphenyl) 2 phenylethane (2×10^{-4})	51	43	48

It is apparent that the inhibition of succinoxidase activity cannot be explained adequately in terms of

the succinic dehydrogenase were practically unaffected by this substance (Fig 2). It is clear that the mechanism of inhibition involves some factor or stage additional to those yet considered.

It was noticed that when cytochrome c was added to the buffered flask contents containing 4 4'-dihydroxystilbene, prior to the introduction of the enzyme preparation, a change of colour suggestive of partial reduction of the cytochrome took place within a few minutes. This was confirmed by the appearance of the absorption bands of reduced cytochrome c (though not in great intensity) under these conditions. If the inhibition of succinoxidase produced by the stilbene derivative depends upon the partial destruction or immobilization of cytochrome, it should be reversed by large increases in the amount of cytochrome available. The experiment recorded in Fig 3 was carried out to test this possibility. The lowest concentration of cytochrome c employed here, viz 3×10^{-5} M, is sufficient to saturate the succinic oxidase system with this component. At a concentration of 4 4'-dihydroxystilbene giving a convenient degree of partial inhibition, there were no significant differences in the oxygen uptake with a two or five fold increase in cytochrome concentration.

Further observations indicate that the inhibitors may affect either the reduction or the reoxidation of cytochrome c. In controls without oestrogen, the cytochrome c at the end of an experiment is always in the oxidized state, it becomes reduced in a few minutes on standing and can be reoxidized by shaking with air. The process is freely reversible. If complete or nearly complete inhibition of succinoxidase has been caused by stilboestrol, hexoestrol,

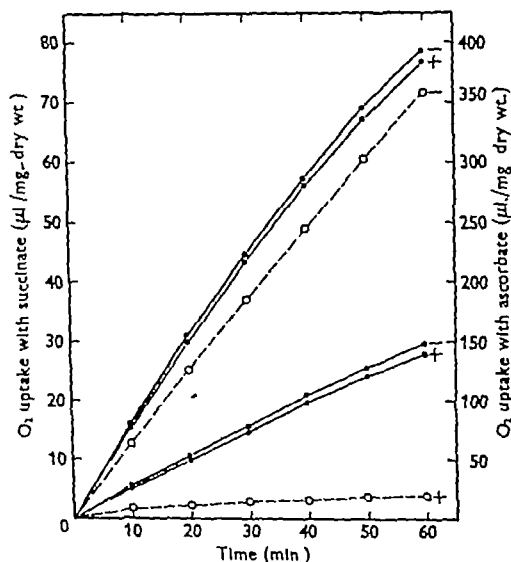


Fig 2 Effect of 4 4'-dihydroxystilbene (2×10^{-4} M) upon cytochrome oxidase (upper pair of continuous curves), succinic dehydrogenase (lower pair of continuous curves) and the complete succinoxidase system (discontinuous curves). Minus sign, without inhibitor, plus sign, with inhibitor. The ordinates indicated on the right-hand side apply to cytochrome oxidase only. Inhibitions (%) succinoxidase, 93, succinic dehydrogenase, 7, cytochrome oxidase, 2.

that of either cytochrome oxidase or succinic dehydrogenase, furthermore, inhibition of the former

isohexoestrol or dienioestrol, the cytochrome *c* is seen to be irreversibly reduced. If only partial inhibition has been produced by one of these compounds the cytochrome is oxidized at the end of an experiment and becomes reduced on standing, as in the controls, but at a slower rate depending upon the concentration of oestrogen. Thus, while increasing concentrations of oestrogen progressively retard the reduction of cytochrome *c*, once this has been accomplished the oestrogen, if in high enough concentration, has the opposite effect of completely preventing its reoxidation. Clearly, therefore, more factors than one are affected by these compounds and the situation is more complex than McShan & Meyer's (1946) explanation would imply.

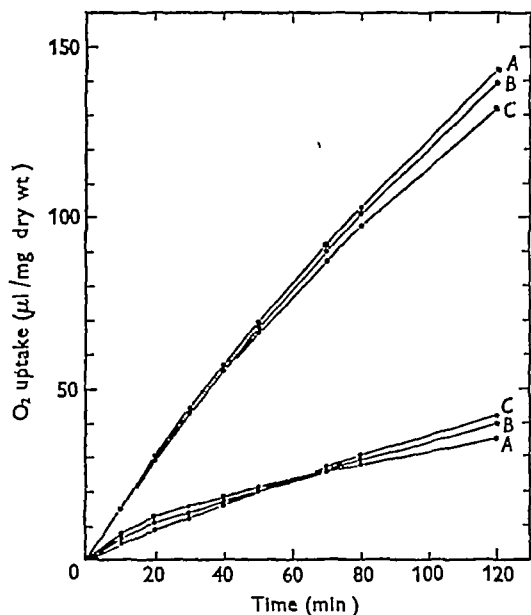


Fig 3 Effect of varying concentration of cytochrome *c* upon the inhibition of succinoxidase produced by 5×10^{-5} M 4,4'-dihydroxystilbene. Cytochrome concentration A, 3×10^{-5} M, B, 6×10^{-5} M, C, 1.5×10^{-4} M. Upper group of curves no inhibitor. Lower group of curves inhibitor present.

The behaviour of 4,4'-dihydroxystilbene is different. This completely inhibits in about the same concentration as does stilboestrol, but in such cases the cytochrome *c* in the reaction mixtures is invariably in the completely oxidized state, and can not be reduced by prolonged standing or anaerobic conditions. Since it has been shown that the succinic dehydrogenase is unimpaired it follows that some factor which links that enzyme to cytochrome *c* has been put out of action.

DISCUSSION

The hypothesis originally put forward by McShan & Meyer (1946), that a relatively simple and specific biochemical property, that of inhibiting a known

enzyme, could be ascribed to all oestrogenic substances, has been shown to be fallacious. On the one hand, there are oestrogens which do not inhibit succinoxidase, and on the other, compounds structurally closely allied, but, nevertheless, non oestrogenic or nearly so, which inhibit it strongly. As already mentioned, McShan *et al.* (1947) have also come to this conclusion. They too encountered slightly oestrogenic and non oestrogenic substances which were highly effective inhibitors, although, because they studied a rather limited series of oestrogens, they did not demonstrate the converse. The fact that they found such substances as thyroxine and hydroquinone to be inhibitors strengthened their opinion that the presence of phenolic groups in the molecule is associated with the property in question. This is probably so, although our results (Table 1) do not support the implication of the American authors that the number of phenolic groups in the molecule bears a direct relation to the ability to inhibit succinoxidase.

We cannot yet explain the absence of inhibitory effect of oestrogens on tissue slices and strips, but considerations of permeability of intact cells or precipitation, destruction or inactivation of the oestrogen by the tissue might all be involved. The appearance of the liver slices after an experiment was sometimes suggestive of a layer of insoluble material. There is evidence in the literature that oestrogens are inactivated by liver *in vivo* and *in vitro*. For example, Heller (1940) demonstrated oxidative inactivation of natural oestrogens by liver slices, Zondek, Sulman & Sklow (1943) extended this observation to stilboestrol, and Zimmerberg (1946) reported that stilboestrol could be inactivated either by conjugation or oxidation. Levy (1947) produced evidence that cytochrome *c* and cytochrome oxidase are implicated in the inactivation of oestradiol by liver.

The main interest of the work that we have reported lies in the question of the precise point or points of attack at which the inhibitors prevent the succinoxidase system in homogenates from functioning normally. Many of them apparently interfere with more than one stage of the enzyme complex, but the existence of a factor linking succinic dehydrogenase to cytochrome *c* is indicated by the inhibition exerted specifically at that point by 4,4'-dihydroxystilbene. Such a suggestion of course has been made frequently during the past decade, and there is little doubt that the classification of succinic dehydrogenase as a 'cytochrome reducing dehydrogenase' (Green & Brosteaux, 1936) is hardly valid without modification. Hopkins, Lutwak-Mann & Morgan (1939) and Stern & Melnick (1939) indicated the probable necessity to postulate a factor able to link succinic dehydrogenase to the cytochrome system. Dixon & Zervas (1939) put forward a similar

claim for yeast lactic dehydrogenase, another so called 'cytochrome reducing' enzyme, Bach, Dixon & Zervas (1946) adduced further evidence that a new factor designated as cytochrome b_2 forms an essential part of this enzyme system, and gave reasons for believing that an additional factor is necessary to gear it to cytochrome c . Keilin & Hartree (1940) referred to the possible existence of an intermediate factor in mammalian succinoxidase, while not excluding the view that integrity of colloidal structures might be the consideration involved. Elliott (1940) believed that a succinoxidase inhibitor which he extracted from tumours and other tissues, and regarded as a proteolytic enzyme, acted upon some entity additional to succinic dehydrogenase and cytochrome oxidase. Straub (1941) was able to sever the linkage between the dehydrogenase and the cytochrome system, and then to remedy the deficiency by means of a heart preparation, thereby reconstituting the whole succinoxidase system. More recently, Stoppani (1947) has shown that a soluble factor can be obtained from liver which is able to link succinic dehydrogenase with cytochrome c . One of the most interesting contributions in relation to the work described in the present paper is that of Ball, Anfinsen & Cooper (1947), who studied the inhibition of a succinoxidase preparation from heart muscle by a series of 2 hydroxy 3 alkyl naphthoquinones. These proved to be extremely potent inhibitors of succinoxidase at even lower concentrations than those requisite with our inhibitors, and were without action upon cytochrome oxidase. However, succinic dehydrogenase, though not the sole or main seat of the effect, was considerably inhibited when functioning with methylene blue, so that although the naphthoquinones used by Ball *et*

al (1947) are undeniably highly useful tools for the elucidation of the sequence of events in succinate oxidation, it may be that 4,4'-dihydroxystilbene, and possibly other compounds of the same type, are capable of yielding clearer results by virtue of their narrower specificity. Very recently Slater (1948) has published a preliminary report of evidence, based on a study of the inhibition of succinoxidase by 2,3-dimercaptopropanol (BAL), which leads to conclusions substantially identical with those expressed here.

SUMMARY

1 The oestrogenic potency of synthetic oestrogens is not correlated with their efficacy as inhibitors of the succinoxidase system of rat liver. Some powerful oestrogens have little or no inhibitory capacity, and the converse is true of many compounds closely related chemically to the oestrogens.

2 The point in the array of respiratory enzymes at which the inhibition takes effect varies from one compound to another. The relative extents to which succinic dehydrogenase and cytochrome c oxidase are inhibited are not the same for different substances.

3 4,4'-Dihydroxystilbene, which possesses low oestrogenic activity, is especially noteworthy because it exerts a powerful inhibitory effect upon the succinoxidase system which is not due to interference either with succinic dehydrogenase or with cytochrome oxidase. The substance is likely, therefore, to be valuable in the further investigation of the respiratory enzyme complex.

4 Further evidence is adduced in favour of the hypothesis that the succinoxidase system involves hitherto uncharacterized factors.

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The Apparent Arginine Content of Human Plasma Proteins by the Sakaguchi Reaction

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The Sakaguchi (1925) reaction consists in the formation of an unstable red compound on treatment of a protein with sodium hydroxide, α naphthol and sodium hypochlorite. It is given by certain guanidine derivatives, notably arginine. Sakaguchi found that the intensities of the colours developed by serum albumin and globulin were approximately equal under the conditions he employed, but no conclusions could be drawn as to the actual arginine content of these unhydrolyzed proteins, since some proteins known to contain very little arginine gave a relatively stronger reaction, weight for weight, than others rich in the amino acid. A modification of the reaction was applied quantitatively to protein hydrolysates by Weber (1930), who substituted sodium hypobromite for hypochlorite and stabilized the colour by adding urea. Weber also observed that the colorimetric determination of arginine in unhydrolyzed proteins was not possible, since the colour values found were much less than would be expected from the actual arginine content (cf also Calvery, 1945). A modification of Weber's hypobromite method was applied to protein hydrolysates by Jorpes & Thorén (1932), who found that the apparent arginine content decreases linearly with increase in the amount of arginine used for analysis; extrapolation to zero concentration would thus be expected to give a close approximation to the true arginine value. Using this technique, which is stated to give results in satisfactory agreement with those obtained by isolation of the amino-acid as the mono- and di-flavianates, Brand and his co-workers (Brand & Kassell, 1942, Brand, Kassell & Sadel, 1944, see also Brand & Edsall, 1947) found the following values for the arginine content of human plasma proteins (in g/100 g of protein): albumin 6.2, γ -globulin 4.8, β -globulin 6.8, α globulin 7.7, fibrin 7.9. Other workers found somewhat lower values by the application of isolation procedures (Block, 1938), e.g. 5.3% for albumin and 4.7% for globulin (Murrill, Block & Newburgh, 1940).

The present investigation was undertaken as a result of the recent publication by Albanese and his co-workers (Albanese & Frankston, 1945, Albanese, Irby & Saur, 1946, Albanese, Saur & Irby, 1947) of a modification of the Sakaguchi reaction which they used for the estimation of unhydrolyzed plasma

proteins. They claimed that when alkaline solutions of plasma proteins were submitted to this modified Sakaguchi reaction and compared with an arginine standard, values were obtained which, when multiplied by a factor, gave figures closely approximating to those obtained by the micro Kjeldahl method. The present findings with plasma proteins differ from those of Albanese and his colleagues in several respects.

METHODS

The Albanese method consists in taking a 1 ml. sample, containing about 3 mg of protein in 2.5N NaOH, adding 5 ml of water and 1 ml of α naphthol (0.1% in 95% ethanol) with vigorous mixing, and allowing to stand for 5 min, then adding 1 ml of 0.06N NaOCl and, exactly 1 min later, 2 ml of 20% (w/v) urea, and finally reading in a photoelectric colorimeter after 5 min. A standard arginine solution is similarly treated. For the differential estimation of the plasma proteins the Na_2SO_4 ether technique of Kingsley (1940) is used. Albanese took a sample of the Na_2SO_4 solution for the 'albumin', and for the 'globulin' determination he dissolved the globulin disk in NaOH and tested a sample.

Carrying out the test, as described by Albanese, a proportionality of colour over the normal range of plasma protein values was not obtained until the hypochlorite concentration was increased from 0.06 to 0.15N†. Even with this concentration, however, there is a slight falling off in proportionality above the upper limits of the normal range of plasma protein values. It is worth remarking that Albanese employed a commercial preparation known as 'Clorox', which might possibly have contained activators of some sort. We used British Drug Houses Ltd. NaOCl solution. (A dilution of 'Milton' gave similar results.) In determining how closely the colour developed was correlated with the N content, we modified the procedure slightly, so as to allow of more precision by avoiding the cumulative error involved in pipetting. To the 1 ml sample of protein in NaOH (in a 10 ml volumetric flask), 4.5 ml of water were added, followed by the other reagents and water to a final volume of 10 ml. The extinction was measured with a Hilger Spekker absorptiometer, with Ilford colour filter 604 (spectrum green). As colour development is influenced by temperature, all tests were carried out in a water bath at 22°.

For total proteins, 0.5 ml of plasma was mixed with 5 ml of water and the proteins precipitated by the addition of 5 ml of 20% (w/v) trichloroacetic acid. After 15 min the proteins were spun down and the tube inverted and drained.

† Dr G. M. Bull, working independently in this School, also found it necessary to increase the NaOCl concentration.

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The protein was then dissolved in 10 ml of 2.5N NaOH at room temperature (No significant amount of NH_4 is lost under these conditions)

For the differential protein estimation, 1 ml of plasma was added to 15 ml of 23% (w/v) Na_2SO_4 , and the mixture shaken vigorously with 6 ml of ether for 2 min. On centrifuging, the 'globulin' collects as a disk at the junction of the water and ether layers. The aqueous ('albumin') layer was decanted, by careful decantation the 'globulin' disk could be made to adhere to the side of the tube. After draining, the 'globulin' was thoroughly washed with 10% trichloroacetic acid, centrifuged, and finally dissolved in 2.5N NaOH (usually 10 ml)

A portion of the 'albumin' filtrate was mixed with trichloroacetic acid and the precipitate spun down. After decanting the supernatant liquid and draining, the 'albumin' was washed with trichloroacetic acid and again spun and drained. Finally it was dissolved in 10 ml of 2.5N NaOH.

In some cases the total protein, 'albumin' and 'globulin' were precipitated not with trichloroacetic acid but with molybdic acid which gave a sharper precipitation.

The washing of the 'albumin' and 'globulin' was necessary to remove Na_2SO_4 , which was found to enhance the colour produced under the conditions of the test. Thus, the addition of 1 ml of 23% Na_2SO_4 increased the extinction

Table 1 Colours produced by the Sakaguchi reaction with (total) plasma proteins

Specimen	Diagnosis	Drum reading (E)	Plasma protein (N x 6.25) (g/100 ml)	Protein in test (mg)	Specific extinction coefficient
Ed	Uraemia	0.586	6.55	3.275	0.1790
Ba	Hyperthyroidism	0.582	6.71	3.355	0.1732
Ru	Gastric ulcer	0.539	6.07	3.035	0.1776
Hu	Sprue	0.409	4.55	2.275	0.1798
Sp	Pneumonia and chronic nephritis	0.600	6.80	3.400	0.1765
Ho	Addison's disease	0.620	7.12	3.560	0.1742
Ju	Haematemesis	0.614	6.87	3.435	0.1787
Ca	Myxoedema	0.584	6.71	3.355	0.1741
Sp	Lung abscess and chronic nephritis	0.588	6.75	3.375	0.1743
Hu	Sprue	0.470	5.25	2.625	0.1700
St	Acute intestinal obstruction	0.619	7.04	3.520	0.1759
Wt	Rheumatoid arthritis	0.623	7.19	3.595	0.1733
Sp	Lung abscess and chronic nephritis	0.635	7.21	3.605	0.1762
Ed	Uraemia	0.574	6.41	3.205	0.1791
Go	Hypertension	0.492	5.64	2.820	0.1744
Gr	General paralysis of the insane	0.527	6.09	3.045	0.1731
Sp	Lung abscess and chronic nephritis	0.593	6.78	3.390	0.1750
Ke	Normal	0.590	6.85	3.425	0.1724
Ab	Normal	0.614	7.20	3.600	0.1705
Va	Normal	0.630	7.24	3.620	0.1740
Ma	Normal	0.580	6.64	3.320	0.1747
					Av 0.1755

Standard deviation 0.0015, coefficient of variation 0.9, maximum deviation from average 2.9%

Table 2 Colours produced by the Sakaguchi reaction with plasma 'albumin' and 'globulin'

Specimen	Diagnosis	'Albumin'/'globulin' ratio	Specific extinction coefficients			
			'Albumin'	'Globulin'	Total	
					Calc	Found
Ha	Ptosis	1.67	0.1580	0.1868	0.1688	—
Gr	General paralysis of the insane	1.73	0.1658	0.1830	0.1720	0.1731
Br	Ulcerative colitis	0.57	0.1612	0.1838	0.1756	—
Ta	Hodgkin's disease (?)	1.02	0.1684	0.1830	0.1706	—
Key	Normal	1.43	0.1654	0.1868	0.1744	0.1724
Gi	Rheumatoid arthritis	1.00	0.1668	0.1846	0.1758	—
Ab	Normal	1.42	0.1624	0.1838	0.1713	0.1705
Va	Normal	1.47	0.1771	0.1744	0.1760	0.1740
Ma	Normal	0.95	0.1726	0.1736	0.1732	0.1747
Am	Hypertension	1.36	0.1710	0.1860	0.1772	—
He	Uraemia and alkalosis	1.15	0.1550	0.1808	0.1670	—
Ur	Normal	1.98	0.1677	0.1840	0.1730	—
Av			0.1651	0.1826	0.1729	0.1727
S.D.			0.0067	0.0030	0.0033	0.0017
Coefficient of variation			4.1	1.6	1.9	1.0
Max. deviation from average (%)			7.3	4.9	3.4	1.0

coefficient by about 8%, whether 0.15 or 0.06N NaOCl was used, a blank test containing Na_2SO_4 being carried out at the same time

In each determination, 1 ml. of the NaOH solutions of total protein, 'albumin' and 'globulin' was taken for the colour test, and 3 ml. for the micro-Kjeldahl

The result was recorded as a specific extinction coefficient, i.e. the extinction coefficient corresponding to 1 mg of protein ($\text{N} \times 6.25$) in the 10 ml. of test solution, in a 1 cm cell

$$\left(\frac{\text{reading with 1 cm cell}}{\text{mg protein in test}} \right)$$

The specific extinction coefficient is thus a measure of the ratio of the apparent arginine content to the total nitrogen

RESULTS

Table 1 shows the results obtained for total proteins in 21 specimens from 16 cases, including 4 normals

Table 2 shows the results obtained for 'albumin' and 'globulin'. It will be seen that the specific extinction coefficients in all but two cases were different for 'albumin' and 'globulin', and also that the coefficient for total proteins appears to be the resultant of those for 'albumin' and 'globulin' (The two cases having the same coefficient for 'albumin' as for 'globulin' were both Indians: one had always been on a vegetarian diet, and the other had been on such a diet until a year previously, though whether this is of any significance is not known)

DISCUSSION

These results do not confirm those of Albanese and his co-workers, who found that the same factor for the conversion of arginine to total protein, namely 19.2, was applicable to 'albumin' and 'globulin' as well. This factor is based on figures for the arginine content of body proteins given by Block & Bolling (1945), but in fact the figures given by those authors do not appear to justify the adoption of this factor for albumin and globulin

The results given above, on the other hand, appear to be more in keeping with the figures of Brand and his colleagues, according to which the arginine content of the 'globulin' fractions, calculated from that of the electrophoretic α , β and γ globulin components of plasma and their relative proportions, is higher than that of 'albumin'. In one case, the specific extinction coefficient for fibrin measured by the present author gave a value of 0.2220. This also is in agreement with Brand's figures, which show a considerably higher arginine content for fibrin than for the other protein fractions

It must be emphasized that the specific extinction coefficient, as defined above, depends not only on the

'arginine' but also on the nitrogen content of the protein, which, according to Cook (1946), is somewhat lower for 'globulin' than for 'albumin'. If it be assumed that the arginine content of serum albumin is the same as that of globulin, then the specific extinction coefficients for these fractions would be expected to be in inverse ratio to their relative nitrogen contents, i.e.

$$\frac{\% \text{ N of albumin}}{\% \text{ N of globulin}} = \frac{15.4}{14.3} = 1.08$$

The ratio ('globulin'/'albumin') of the average specific extinction coefficients found by us is $0.1826/0.1651 = 1.105$. The ratio of the arginine content of (electrophoretic) globulin and albumin according to Brand's figures is 1.06

These results appear to suggest that the colour intensity developed under the conditions employed is proportional to arginine content even in unhydrolyzed proteins, but it is deemed advisable to refer to 'apparent arginine' content as the matter cannot be regarded as settled. In view of Brand's figures, and those reported here, the claims for an identical arginine:nitrogen ratio for albumin and globulin cannot be accepted

In view of the well-marked effect of sodium sulphate on colour development, and the possibility of other substances also interfering, it is evident that this reaction should not be applied uncritically to the determination of arginine in biological fluids

SUMMARY

1 The technique of Albanese & Frankston (1945) for the determination of plasma proteins by a modified Sakaguchi reaction gave a proportionality of colour over the normal range of values only when the hypochlorite concentration was increased to 0.15N

2 A 'specific extinction coefficient' (measure of ratio of apparent arginine to total N) has been measured for total plasma proteins in 21 specimens from 16 cases

3 In 10 out of 12 cases in which sodium sulphate-separated 'albumin' and 'globulin' were separately examined, the specific extinction coefficient for 'globulin' was, on the average, 13% higher than that for 'albumin'

4 Both with 0.06N and with 0.15N sodium hypochlorite, the presence of sodium sulphate led to a large increase in the amount of colour developed

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Amino-acid Metabolism of Tissue Cells *in vitro*

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Ever since the negative results of the investigations by Burrows & Neymann (1917), Carrel & Ebeling (1924) and Baker & Carrel (1926, 1928) the amino acids have been regarded as being able neither to prolong the life of cells nor to accelerate cell growth in tissue cultures. However, in these investigations normal culture media were used and such media already contain the necessary amino acids. This point was realized by the present author, and a technique was devised (Fischer, 1941) whereby the compounds of the culture medium were dialyzed against a Ringer's solution containing glucose. It was found that the dialyzed culture medium was completely unable to maintain the life of the cells—they died and disintegrated within 24 hr. With this dialyzed medium as a basis it became possible to make a systematic analysis of the effect of substances of low molecular weight on the maintenance of cells grown *in vitro*.

A study was then made of the effect of an amino acid mixture composed of nine amino acids in the same relative proportions as found by Bergmann & Niemann (1936) in fibrin (Table 1). It was found that cystine plays a very important part. Without cystine, the remaining amino acids are no longer able to save the cells from rapid disintegration within 24 hr. It was also observed that in the cultures of myoblasts and osteoblasts cystine cannot be replaced by methionine, though methionine is an essential acid for the organism as a whole whilst cystine is not. Moreover, both lysine and glutamic acid were found to be of great importance to the cells. Obviously, the amino acids required by the whole organism are not the same as those necessary

to a pure culture of tissue cells. For example, glutamic acid is essential to myoblasts, but not to the organism as a whole. Our technique of 7 years ago has since been improved, and the present paper presents some of the results obtained.

EXPERIMENTAL

The tissue cells in the present experiments belonged to a pure strain of myoblasts from a 9 day-old chicken embryo. The individual cultures were divided into two halves, one serving as control in the medium described below, the other as the experimental culture, in the same medium, but with the substance under investigation. At intervals during growth, drawings of the tissue cultures were made by means of an Edinger projector which magnified twenty times. The area of each drawing was measured by means of a planimeter and the results are given in Figs 1-14, expressed as ratios according to the formula $(B-A)/A$, where A =initial area and B =growth area (Fischer, 1925).

The medium consisted of 0.5 ml dialyzed chicken plasma, 1 ml Tyrode's solution and 0.1 ml of the mixture, called the basic nutrient (Table 1). Coagulation was produced by adding 1 drop of dialyzed embryo juice. When coagulated, a liquid phase was introduced by adding 0.5 ml. dialyzed serum and 0.1 ml. of the basic nutrient. Carrel flasks type D 3 were used. The pH of the mixture was adjusted by introducing into the flask a gas composed of 8% CO₂, 12% N₂ and 80% O₂. The flasks were sealed with sterile rubber stoppers.

The basic nutrient mentioned (Table 1) was designed by G Ehrensward, and is a mixture of substances known empirically to be important to the whole organism. It contains three main groups of substances, the first group consists of salts including salts of heavy metals and organic phosphates which can function as phosphorylating agents,

Table 1 *Basic nutrient of biologically active substances tested in tissue cultures*

(Mg of substances contained in 1 l solution.)

NaCl	7500
KCl	200
CaCl ₂	200
MgCl ₂	100
Na ₂ HPO ₄	50
NaHCO ₃	1000
FeCl ₂	0.6
CuCl ₂	0.2
MnCl ₂	0.3
ZnCl ₂	1.0
CoCl ₂	0.01
Glucose	800
Mannose	100
Galactose	100
Inositol	20
Adenosine triphosphate	200
Fructosediphosphate	100
β Glycerophosphate	100
Inosinic acid	30
Cozymase	5
Aneurin	3
Riboflavine	0.2
Pyridoxin	0.3
Lysine dihydrochloride	15.1
Arginine monohydrochloride	7.7
Tryptophan	5
Methionine	2.6
Histidine monohydrochloride	3.1
Glutamic acid	14.1
Aspartic acid	5.9
Proline	5.1
Cystine	1.5
DL Methionine	6
Choline (as hydrochloride)	10
Creatine	10
Nicotinic acid	0.3
Glutathione	5
Pantothenic acid	0.07
Biotin	0.007
p Aminobenzoic acid	1
Hypoxanthine	100
Sodium succinate	10
Sodium fumarate	10
Sodium malate	10
Sodium oxaloacetate	10
Ascorbic acid	2
Methylnaphthohydroquinone sulphate	0.005

another group comprises the amino acids, substances acting as methyl donors or providing SH groups, and finally a third group containing the various vitamins, choline, creatine and the C₄ acids (functioning in the Krebs cycle) This basic nutrient is so designed that it is easy to eliminate any part of it and replace it by another

RESULTS

Importance of amino acids as a group

The first and fundamental experiment involved the question of the general importance of the amino-acids in connexion with the maintenance and growth of tissue cells. It was found, as one would expect, that if no amino acids are present in the basic nutrient the cells die and disintegrate rapidly (Table 2). To be sure, there is a very slight growth on the first day, but that is due to residual amounts of amino acids in the tissue itself at the time of transfer to the dialyzed, incomplete medium. The importance of the individual amino acids could now, as mentioned above, be studied by omitting the amino acid in question from the mixture.

Deficiencies of single amino-acids

Cystine The absence of cystine and methionine in animal nutrition leads to death in a comparatively short time but, since the myoblasts are unable to utilize methionine in place of cystine, the withdrawal of this amino-acid from our medium has no effect on the growth of the cells. Cystine forms a structural constituent of the proteins of the cell. Fig. 1 and Table 3 show that the control culture in the medium containing the whole of the basic diet grows normally, whereas the experimental culture, with no cystine in the medium, does not grow at all. This confirms our earlier findings (Fischer, 1941) as to the basic significance of cystine. It was also found that methionine, in the absence of cystine, is insufficient (Fig. 1). When cystine was added to the deficient medium, the cells not only survived, but showed a small but definite increase in mass, and the cells had a normal appearance.

Cystine can, however, be replaced by glutathione (Fig. 2). It is believed that in addition to being of

Table 2 *Comparison of growth of myoblasts on the basic nutrient with and without amino-acids*

Exp. no.	Days	Superficial growth in medium with amino acids (mm ²)	Superficial growth in medium without amino acids (mm ²)	Ratio of area of growth with and without amino acids
18213-14	7	855	86	9.9
18215-16	7	1267	154	8.2
18217-18	7	1076	95	11.3
18219-20	7	1096	191	5.7
18221-22	7	1116	98	11.3
18257-58	5	—	—	—
18259-60	5	—	—	—
17967-68	4	1051	190	5.5
17969-70	4	1005	212	4.7

importance as a building stone for the cytoplasm, cystine is also an activator for various proteolytic enzymes which enable the cells to split the plasma

proteins of the medium, or possibly to the fact that glutamine, together with cystine and glycine, may enable the cells to produce other amino acids for their maintenance

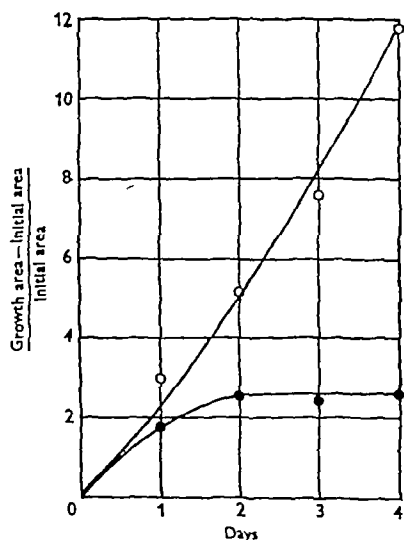


Fig 1 Effect of cystine deficiency on growth of myoblasts. The circles indicate the growth in the control medium, the black dots the growth when cystine is omitted.

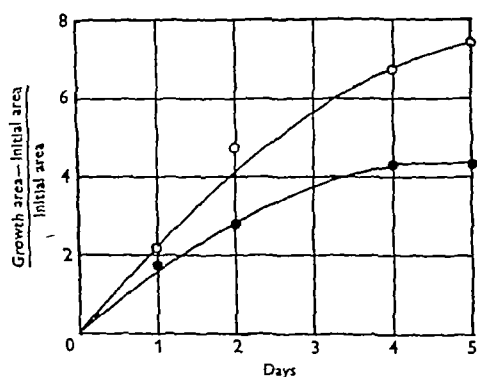


Fig 2 The effect of the replacement of cystine by glutathione. The black dots indicate the growth in the control medium, the circles the growth when cystine is replaced by glutathione

proteins in the surrounding medium. The break in the curve in Fig 3 may be explained as being due to a liberation of split products from the plasma

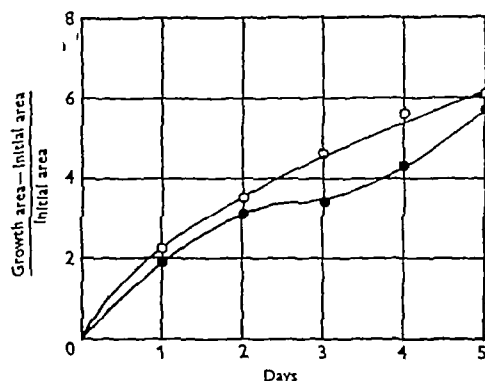


Fig 3 Effect of a medium containing only cystine, glycine and glutamine. The circles indicate the growth in the control medium, the black dots the growth when the amino acids comprise only cystine, glycine and glutamine

Glutamic acid is known to be very important and to be associated with the transamination process. According to Rose (1937) this amino acid has no effect on the growth rate of young rats. The growth of myoblasts on a nutrient without glutamic acid was slightly retarded in comparison with that shown with the basic diet (Figs 4, 9). If glutamic acid was replaced by glutamine (0.5 mg/flask) the rate of growth increased enormously (Fig 5). At the same time, the morphological appearance of the cells underwent a change and became perfectly normal, and gradually the fat vacuoles disappeared. Without glutamine the cells were extremely atrophic, having the appearance of small glass splinters. The process is reversible, i.e. cells which already are highly atrophic change into perfectly normal cells when glutamine is added to the medium.

Lysine. The absence of lysine from the amino acid mixture had only a slight effect or none at all on the growth of myoblasts (Fig 6), lysine thus resembles glutamic acid (Fig 4). This may be connected with the circumstance that lysine cannot be regenerated by amination once it is deaminated (cf. Weissman & Schoenheimer, 1941). Osteoblasts seem to be more sensitive to lysine deficiency (Fig 7).

Table 3 Growth of fibroblasts on the basic nutrient with and without cystine

Exp no	Days	Superficial growth with cystine (mm ²)	Superficial growth without cystine (mm ²)
18257-58	5	614	0
18259-60	5	805	0
18269-70	4	700	0
18271-72	4	571	0

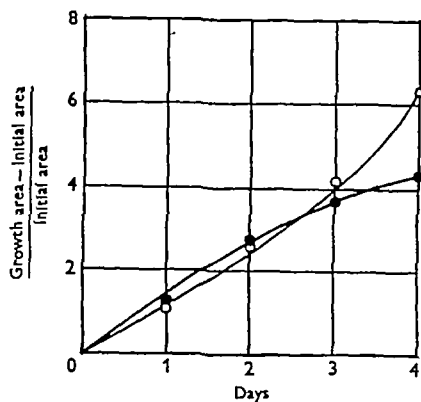


Fig 4 Effect of a deficiency of glutamic acid on the growth of myoblasts (Exp 18261-62) The circles indicate the growth in the control medium, the black dots the growth when glutamic acid is omitted.

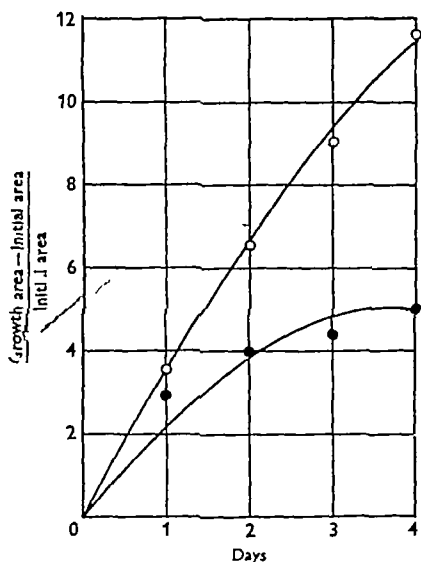


Fig 5 Comparison of the effect of glutamine and glutamic acid (Exp 18291-92) The black dots indicate the growth in the control medium, the circles the growth when glutamic acid is replaced by glutamine

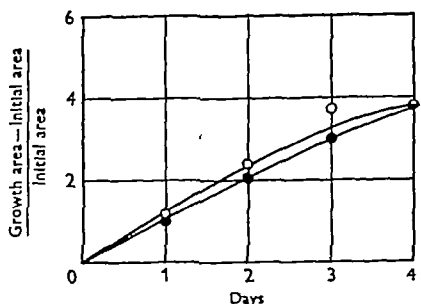


Fig 6 Effect of lysine deficiency on the growth of myoblasts (Exp 18375-76) The circles indicate the growth in the control medium, the black dots the growth when lysine is omitted. There is practically no difference

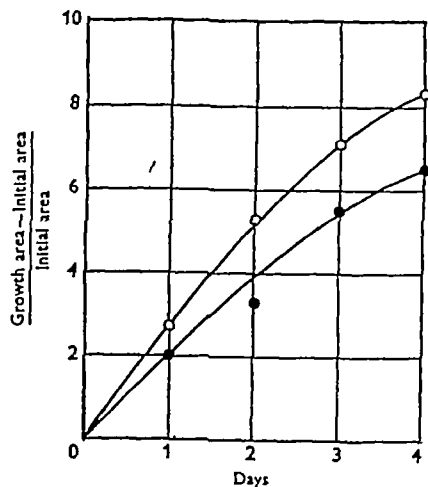


Fig 7 Effect of lysine deficiency on the growth of osteoblasts (Exp 18527-28) The circles indicate the growth in the medium containing all the amino acids of the basic diet, the black dots the growth in the medium when lysine is omitted

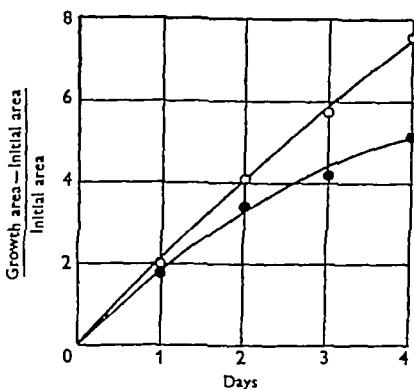


Fig 8 Effect of the combined deficiency of lysine and glutamic acid on the growth of myoblasts (Exp 18479-80) The circles indicate the growth in the control medium, the black dots the growth when both lysine and glutamic acid are omitted

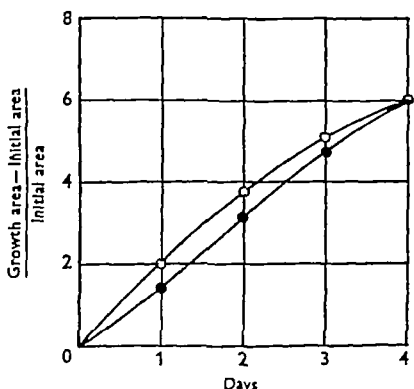


Fig 9 Effect of glutamic acid deficiency on growth of myoblasts (Exp 18263-64) The circles indicate the growth in the control medium, the black dots the growth when glutamic acid is omitted

A medium lacking both lysine and glutamic acid has a growth depressing effect (Fig 8), which is more marked than if only one of these amino acids is lacking (Figs 4, 6 and 9). The morphological appearance of the cells was perfectly normal in the control, whereas in the lysine deficient medium the cells were atrophic and of the above mentioned glass splinter type.

Tryptophan According to Osborne & Mendel (1914) tryptophan is an essential amino acid, it may be deaminated *in vitro* under aerobic conditions by slices of kidney (Krebs, 1933). Fig 10 shows the

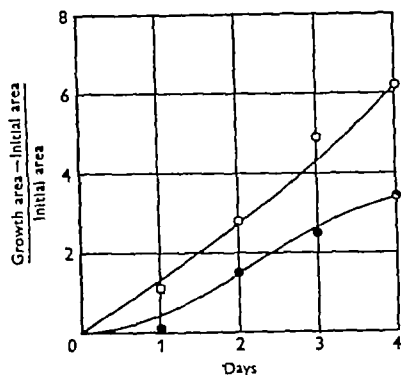


Fig 10 Effect of deficiency of tryptophan on growth of myoblasts (Exp 18379-80). The circles indicate the growth in the control medium, the black dots the growth when tryptophan is omitted.

effect of a nutrient deficient in tryptophan (Exp 18379-80). The response of the cells was plain, in the control medium the cells were perfectly normal, while they showed atrophy in the deficient medium. Morphological differences between the cells on the two diets became manifest from the time the cells began to migrate out into the medium.

Arginine is claimed to be a dispensable amino acid as far as the animal organism is concerned (Scull & Rose, 1930). According to Klose, Stokstad & Almquist (1938) the young chick seems to lack any ability to synthesize arginine. Since the absence of arginine causes an inhibition of the growth of myoblasts, this amino acid must be indispensable to these particular cells which cannot, like the mammalian organism, synthesize arginine (Moss & Schoenheimer, 1940). Fig 11 shows the pronounced effect of arginine deficiency. The cells in the arginine free medium contained, curiously enough, less fat vacuoles than did the control cultures.

Histidine proline Rose & Cox (1924, 1926) have shown that histidine is indispensable to the growth of rats. Removal of proline from the diet is claimed to have no effect on the growth of young rats. Histidine proline deficiency in the basic nutrient led to a marked depression of the growth of our experimental cultures (Fig 12). The cells in these cultures

were thin and atrophic, containing numerous fat vacuoles, whereas the cells in the control cultures, with all the amino acids in the medium, were perfectly normal.

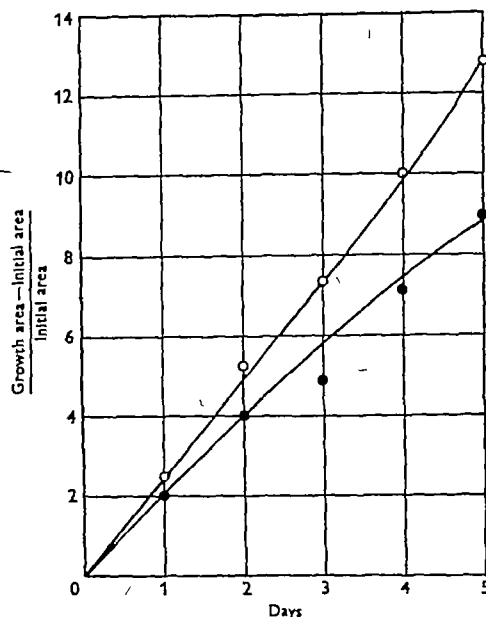


Fig 11 Effect of arginine deficiency on growth of myoblasts (Exp 18437-38). The circles indicate the growth in the control medium, the black dots the growth when arginine is omitted.

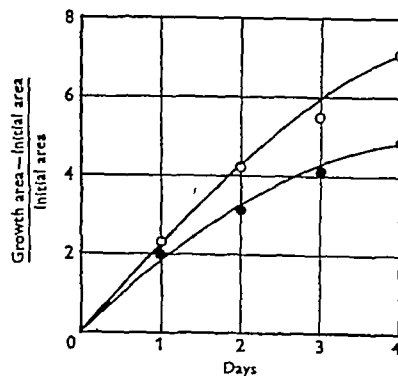


Fig 12 Effect of combined deficiency of histidine and proline on growth of myoblasts (Exp 18555-56). The circles indicate the growth in the control medium, the black dots the growth when histidine and proline are omitted.

The effect of media containing only few amino acids

Medium containing only cystine, histidine, proline and aspartic acid The growth of myoblasts in a medium, the amino acids of which include only these amino acids, was, as one would expect, very defective. There was practically no growth, the cells

were full of fat vacuoles, and disintegrated very rapidly

Medium containing only cystine, glutamine and glycine A medium containing only these amino acids and all the other components of the basic diet showed a remarkable effect. The concentration of each of the components was the same as in the basic nutrient and 0.5 mg glutamine was used in each flask. The cells looked perfectly normal, and no difference could be observed between the cells in this experimental medium and the cells in the control. Cystine alone was not capable of keeping the cells alive for any length of time (Exp 18339-43). It is thought that the cells might be able to build other amino acids by transamination of the three amino acids mentioned.

Amino acids and digests of proteins

Complete chemical analysis of several proteins has recently been undertaken by Brand (1946). These data make it possible to carry out a comparative investigation of the effect on tissue cells of, on the one hand, an amino acid mixture approximating the composition of lactoglobulin and crystalline bovine serum albumin and, on the other, a peptic and tryptic digest of the same proteins. For the preparations of lactoglobulin and pure trypsin we are indebted to Prof Linderstrom Lang. Armour's crystalline pepsin was used.

The crystalline enzymes were first dialyzed for 24 hr. The proteins to be digested were diluted with water 1:3 and toluene was added to prevent bacterial growth. The digestion was followed by heating for 5 min on the water bath at 100°, cooling, filtration, evaporation *in vacuo* to remove the toluene. pH was adjusted by means of NaOH or HCl, depending on the enzymes used. The solutions were sterilized by filtration.

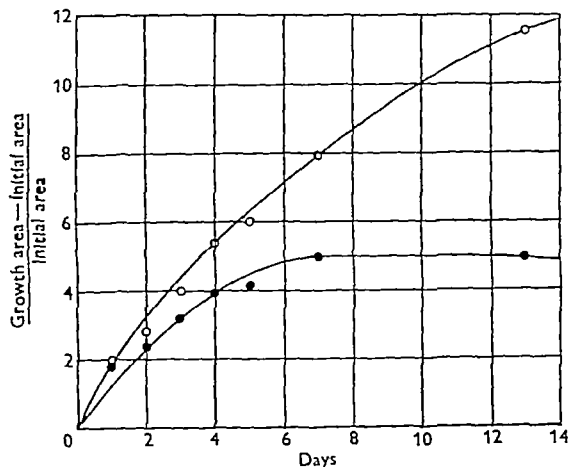


Fig 13 Effect of a digest of lactoglobulin and an artificial amino acid mixture resembling this protein (Exp 18233-34). The black dots indicate the growth in the amino acid medium, the circles the growth when the medium contains the enzymic digest of lactoglobulin.

Figs 13 and 14 show the complementary effects on the deficient plasma medium of a mixture

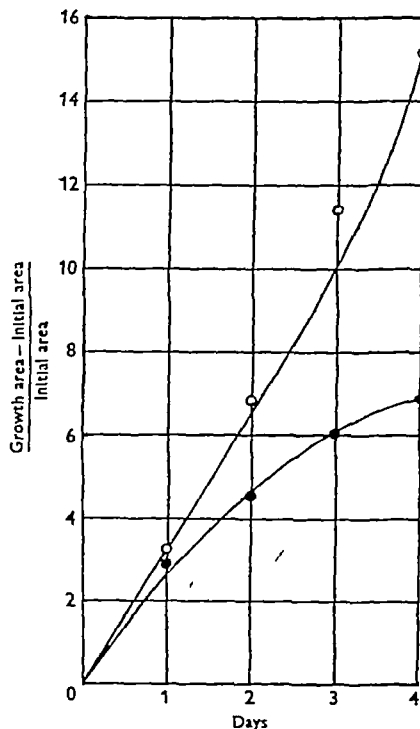


Fig 14 Effect of a digest of bovine serum albumin and of an amino acid mixture resembling this protein (Exp 18455-56). The black dots indicate the growth in the amino acid medium, the circles the growth when the medium contains the bovine serum albumin digest.

Table 4 *Amino-acid composition of lactoglobulin and bovine serum albumin*

Amino acids	Lactoglobulin (mg in 20 ml.)	Bovine serum albumin (mg in 20 ml.)
Glycine	1.4	1.9
Alanine	6.2	—
Valine	5.83	6.5
Leucine	15.6	13.7
Isoleucine	8.4	2.9
Proline	4.1	5.7
Phenylalanine	3.54	6.2
Cysteine	1.11	1.11
Cystine	2.29	5.41
Methionine	3.22	0.81
Tryptophan	1.94	0.58
Arginine	2.88	6.2
Histidine	1.58	3.8
Lysine	11.4	12.4
Aspartic acid	11.4	10.6
Glutamic acid	19.5	16.9
Glutamine	1.5	1.05
Serine	5.0	4.5
Threonine	5.85	6.5
Tyrosine	3.78	5.49

of the amino-acids approximating in their relative proportions to the composition of the proteins, lactoglobulin and bovine serum albumin (Table 4).

The effects were pronounced. While the proteins in the form of amino acids produce an excellent complementary effect on the deficient plasma medium, the digests cause besides an enormously increased rate of growth, a phenomenon known already from the work of Baker & Carrel (1928) when using proteoses from Witte's peptone.

For the same nitrogen content there is a remarkable difference in the effects of the amino acids and the digests of the lactoglobulin. While the growth curve of tissue cells in a medium containing the amino acids slowly approaches a maximum, the growth of the cells in the medium containing the digests proceeds almost logarithmically from the very beginning and reaches high values (Figs 13, 14). In other words, the cells respond quite differently to amino acids and to polypeptide amino acid mixtures.

DISCUSSION

Comparative experiments on the importance of amino acids in the nutrition of pure strains of tissue cells *in vitro* demonstrate the significance of the individual amino acids as far as growth and maintenance are concerned. Cystine occupies a key position and was found to be the only amino acid, the absence of which leads to complete inhibition of growth even in the presence of all the other amino acids, a fact which was already recognized by the author several years ago (1941) when he employed a nutrient composed of amino acids only. Since then we have developed a complete basic nutrient containing all the substances necessary for satisfactory growth. Here again it has been confirmed that cystine cannot be replaced by methionine, as myoblasts are found to be unable to metabolize this amino acid under the experimental conditions employed.

It is concluded that those amino acids, which when withdrawn from the mixture cause a depression of cell growth, are normally metabolized by the cells in question and may therefore be regarded as indispensable to these types of cells.

The experiments also show that a nutritive evaluation of the individual amino acids on the basis of their effect on strains of tissue cells is very rapid in comparison with other methods involving whole

animals. For the investigation of the protein metabolism of cell types, the tissue culture method is the only one that can be used. It is evident also that the amino acid requirement of various types of tissue cells may not be the same, as is shown by a comparison of the response of myoblasts and osteoblasts to lysine deficiency (Figs 6, 7). Perhaps we may soon be in a position to map the amino acid diet necessary for the maintenance of other types of tissue cells.

An approximate rating of the amino acids according to the degree to which they are essential to the cells of the cultures in the present experiments is as follows: cystine (most essential), arginine, tryptophan, glutamine and lysine.

SUMMARY

1 A basic nutrient, however elaborate, involving compounds found empirically to be of importance to the animal organism is inadequate for the maintenance and growth of tissue cells if it does not contain the necessary amino acids.

2 A technique has been developed making it possible to evaluate accurately the importance of each of the amino acids necessary to the life of the cells.

3 If the elimination of an amino acid from the diet causes the inhibition of cell growth, in comparison with the growth of the control culture, this amino acid must be, when present, metabolized by the cells and must be regarded as indispensable.

4 The degree to which an amino acid is essential can be determined accurately by measuring the ratio between the growth of the controls in the medium containing all the amino acids and the growth of the experimental cultures where the medium is deficient in this particular acid. The present experiments involved cystine, methionine, lysine, glutamic acid, aspartic acid, tryptophan, arginine, glutathione, histidine and proline.

5 The effect of the amino acids constituting lactoglobulin and crystalline bovine serum albumin was compared with that of enzymic digests of these proteins. The differences are very marked and characteristic.

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Studies in Penicillin Production by *Penicillium notatum* in Surface Culture

2 FURTHER STUDIES IN THE METABOLISM OF SULPHUR

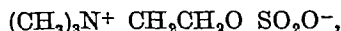
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(Received 24 March 1948)

An earlier report from these laboratories described the metabolism of carbon and nitrogen compounds by *Penicillium notatum* in surface culture (Hockenhull, 1946), and some references were made to the utilization of sulphur by this organism. Further work on the latter aspect seemed desirable, especially as penicillin is a sulphur containing compound. It is the purpose of the present communication to describe these investigations.

Previous work on the sulphur metabolism of moulds is limited and is mainly concerned with *Aspergillus niger*. Rippel & Behr (1936) showed that this fungus, when supplied with sulphates, liberated considerable quantities of organic sulphur compounds into the medium. Möthes (1938) showed that when cystine, cysteine or thioglucose was supplied sulphates appeared in the medium. Möthes also showed that the reduction of sulphate to organic sulphur was greater in presence of ammonia than in presence of nitrate. Steinberg (1941) showed that many inorganic sulphur sources were available for the growth of *A. niger* (e.g. sulphate, thiosulphate, etc.) and concluded that the mould removed sulphur from organic compounds via sulphylic acids. Woolley & Peterson (1937) demonstrated the accumulation of 'cyclic' choline sulphate (β sulphato ethyltrimethylammonium betaine),



in the mycelium of *A. sydowii*.

Penicillium notatum is known to utilize cysteine, cystine or sulphate to produce optimal yields of penicillin. In our experiments we have examined the growth of this organism on a wide range of sulphur sources. The growth of two X ray mutants, which are unable to grow on sulphate, was also examined. It was hoped that a study of these organisms would throw light on the metabolic stages used by the organism in the formation of organic sulphur compounds. Analytical studies were also made on the utilization of sulphur by the mould, and experiments similar to those described by Rippel & Behr (1936) and by Möthes (1938) were also carried out with

P. notatum in order to determine whether their behaviours were the same or different.

EXPERIMENTAL

Cultural methods and materials

Strains of the mould Five strains were used in our experiments: (1) 1249 B21, which was received from the Northern Regional Research Laboratory, Peoria, Illinois, it has been used for the commercial production of penicillin and the culture used by us has been labelled by us M2, (2) F4, received from Sir Howard Florey, it was used at one time for the production of penicillin, (3) 832, received from Peoria, and used for early work in submerged culture, (4) W3 cyst, an X ray mutant of Sir Alexander Fleming's strain, which, although white and powdery, also produces a yellow pigment freely on most media, (5) 832A, an X ray mutant of strain 832 morphologically similar to its parent strain, to which it tends to revert occasionally. The last two strains were received from Dr G. Pontecorvo of Glasgow University to whom our thanks are due. They both grow freely on media containing cystine but, unlike the others, do not grow on media containing sulphate as sole source of sulphur.

Growth conditions All experiments with liquid media were carried out using 250 ml. conical flasks, with 100 ml. of medium in each. The medium was steam sterilized for 20 min. at 15 lb./sq. in. throughout.

The inoculum was a spore suspension containing 3×10^7 spores/ml., based on direct counts with a haemocytometer cell. It was prepared by washing off spores from cultures on glycerol molasses peptone (cf. Moyer & Coghill, 1946) with sterile water. 1 ml. of this suspension was pipetted into each culture vessel. All cultures were incubated, stationary, at $23.5^\circ \pm 0.5^\circ$.

Media The media most commonly used were synthetic and were based on a 'standard basal' medium (hereafter referred to as SB medium) which had the following composition (as % w/v): lactose (B.P.), 3, glucose (B.P.), 1, citric acid (B.P.), 1.5, acetic acid, 0.25 (v/v), phenylacetic acid (re-crystallized), 0.05, source of N (as text), source of S (as text), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.05, FeCl_3 , 0.005, $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$, 0.002, ZnCl_2 (fused), 0.002, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.002, $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, 0.002, NaH_2PO_4 , 0.1, NaF, 0.001.

The medium was made in tap water and was adjusted to pH 5.8-6.0 with 56% (w/v) KOH before sterilization. It was modified quantitatively or qualitatively by varying either the N source or the S source, or both. Unless

otherwise stated, all ingredients were A.R. quality. Other media employed are described in the text. 'SB agar' was similarly constituted except for the incorporation of 2.5% powdered agar.

Analytical procedure

Sampling Five replicate flasks were removed at the appropriate times. Sterile samples were withdrawn and diluted twenty times with pH 6.5 phosphate buffer (0.05M) for penicillin assay. The mycelium was separated by filtration, and washed with five separate lots of 100 ml. distilled water. The pH and volume of first filtrate were measured. The washings, which were found adequate for complete removal of soluble matter and medium from the felt, were added, together with 10 ml. of conc. HCl, and the whole made up to 1 l. less an allowance for the medium used for penicillin assay. The solution was stored at 0°. In experiments with cystine and cysteine the filter paper was separated from the mycelial mat and was analyzed for precipitated S compounds.

The mycelium was dried to constant weight at 80°, weighed, powdered and stored for analysis. The composition of the mycelium with respect to % N or % S on dry wt was found to be the same if the undried mycelium was homogenized with 20 vol. of distilled water, thus indicating that there was no loss of N or S on drying.

Total N was estimated by the micro Kjeldahl method.

Total S was determined by the method of Luke (1943) but using a different mechanical procedure. Wet combustions of a sample containing 3–5 mg. of S were carried out in a silica crucible with a HNO₃, Br₂ solution containing dissolved ZnO. The subsequent reduction and distillation of H₂S was performed in a 50 ml. flask fitted to a spiral condenser. A stream of N₂ was passed through the apparatus and the H₂S was trapped in a spiral bubbler containing 10 ml. of the alkaline CdCl₂ reagent. After all the H₂S had been absorbed 10 ml. of 1.5N HCl solution containing 3 ml. 0.1N I₂ were added to the bubbler and quickly mixed. The H₂S was estimated by back titration with 0.01N Na₂S₂O₄.

An alternative procedure used for checking purposes was the combustion of the same quantity of material using Benedict's reagent (Cole, 1933), followed by estimation of sulphate with benzidine. This method was found to be less accurate than the other.

Sulphate Solutions containing 3–4 mg. of sulphate S were adjusted with HCl or NaOH until slightly acid to congo red paper. The sulphate was precipitated as benzidine sulphate (Cole, 1933). The precipitate was collected in a 30 mm. Gooch crucible containing asbestos wool. An alternative but more tedious method was precipitation with BaCl₂ in acid solution, followed by reduction of the BaSO₄ to H₂S as in the estimation of total S.

'Bound sulphate' in the mycelium was estimated by hydrolyzing about 300 mg. of dried mycelium at 15 lb./sq. in. steam pressure for 2 hr. with 2.5N HCl. The liquor was made up to 25 ml. and filtered. 10 ml. were taken for estimation of sulphate by the BaCl₂ method.

Cystine This was estimated by the procedure of Shino-hara (1935). It was found that during medium preparation all cysteine was converted to cystine or oxidized further. Both cysteine and cystine media were therefore estimated by the cystine method.

Penicillin Penicillin was estimated by the plate assay methods in current use in these laboratories using *Bacillus subtilis* as the test organism (cf. Foster & Woodruff, 1944), and expressed as Oxford units.

RESULTS

Three groups of experiments were carried out. In the first various strains, including the irradiated strains, were inoculated on to media containing a variety of sulphur sources, and growth was observed in each case. The availability of different forms of combined sulphur was determined in this way. Mixtures of the two irradiated strains were also inoculated on to sulphate media. In the second group synthetic media which yield penicillin were metabolized by strain 1249 B21 and the utilization of sulphur examined at intervals. In the third, some of the experiments carried out by earlier workers with *Aspergillus niger* were repeated using *Penicillium notatum* strain 1249 B21.

(1) Availability of different sulphur sources to normal and irradiated strains of *Penicillium notatum*

Different sulphur sources

The five strains of the mould were grown on SB medium to which were added 0.5% of NH₄NO₃ and one of a large number of sulphur compounds. 1 mg./ml. of the sulphur compound was added. Three replicate flasks were used. The sulphur compound was dissolved in water and sterilized by autoclaving (or by Seitz filtration in the case of Na₂S and Na₂S₂O₄) and added to the sterile medium. Growth is shown as dry mycelial weight expressed as a percentage of the dry weight obtained using medium containing L-cystine. The results of this experiment are shown in Table 1. No growth was obtained unless a sulphur source was added to the basal medium.

Growth of mixed cultures of the irradiated strains

(a) **Liquid media** Media containing as sulphur source sulphate, thiosulphate, hyposulphite, 'cyclic' choline sulphate and acetone sodium bisulphite were inoculated with spore suspensions containing spores of the W 3 cyst and 832A strains or of a mixture of the two. There was no evidence of symbiosis between the two strains which grow satisfactorily only on the media containing hyposulphite and thiosulphate. The irradiated strains were also inoculated into Seitz filtered SB medium containing sulphate, on which strain 1249 B21 had been growing for 4 days. There was no growth, showing that sufficient utilizable sulphur compounds had not been liberated into the medium by strain 1249 B21.

Table 1 *Effect of different sulphur sources on the growth of various strains of Penicillium notatum*(Medium *SB* + S source + 0.5% NH_4NO_3)

Strain of mould	<i>M</i> 2		<i>F</i> 4		<i>W</i> 3 cyst (‘cystineless’)		832		832 <i>A</i> (‘cystineless’)	
Sulphur source (at 1 mg/ml.)	Wt *	Pen †	Wt *	Pen †	Wt *	Pen †	Wt *	Pen †	Wt *	Pen †
Na_2SO_4	100	70	100	12	0	0	102	0	0	0
NaSO_3NH_2	100	58	105	12	0	—	85	15	0	—
$\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$	60	66	100	10	0	—	148	26	20	0
$\text{Na}_2\text{S}_2\text{O}_4$	120	60	114	14	108	0	190	22	108	9
$\text{Na}_2\text{S}_2\text{O}_3 \cdot 10\text{H}_2\text{O}$	100	58	99	19	74	0	148	25	130	23
Na_2S	109	71	94	13	66	0	89	14	51	0
Na_2S_2	—	—	—	—	—	—	—	—	88	12
L-Cystine	100	52	100	14	100	5	100	24	100	20
L-Cysteine HCl	100	58	105	13	98	3	97	22	73	21
Cysteic acid	91	6	98	5	100	0	92	5	92	—
Taurine	100	24	108	12	89	10	94	17	113	15
2-Mercaptoethylamine	80	21	88	—	40	—	124	—	134	—
DL-Dimethylcysteine	40	—	23	—	0	—	50	—	56	—
Mercaptosuccinic acid	50	—	29	—	0	—	55	—	59	—
Thioacetamide	135	—	67	—	56	—	80	—	76	—
Thiourea	33	—	27	—	0	—	57	—	26	—
‘Cyclic’ choline sulphate	125	76	100	16	0	—	94	24	0	—
Acetone sodium bisulphite	100	33	98	10	0	—	100	13	38	—
Ethyl xanthate	123	—	95	—	58	—	102	—	129	—
KCNS	115	—	93	—	40	—	150	—	100	4
Dry weight of felt grown with L-cystine (g/100 ml. medium)	0.65		1.31		1.22		0.57		0.91	

* $\text{Wt} = \frac{\text{mycelial dry weight} \times 100}{\text{mycelial dry weight with cystine}}$

† Pen = Penicillin (units/ml)

(b) *Solid media* 0.1% of Na_2SO_4 was added to *SB* agar medium and 10 ml portions were placed in Petri dishes. Strains *W* 3 cyst and 832 *A* were inoculated on this medium either separately or together.

From the mixed cultures a new type of quickly growing colony developed which could be readily subcultured on the same medium. When single spores from this culture were transferred to *SB* medium containing cystine, the two parental strains were obtained. When hyphal tips were transferred to malt-wort agar, colonies were obtained which sectorized, and from the sectors the parental strains were again recovered. There was thus the appearance of a symbiosis of an interesting type, but further work is necessary before more extensive discussion is possible.

(2) *Metabolism of sulphate in synthetic media on which penicillin is produced*

Reduction of sulphate in SB medium

(a) *SB* medium containing 0.5% $(\text{NH}_4)_2\text{SO}_4$ and 0.5% NaNO_3 was inoculated with strain *M*2 and incubated. Analyses showed that only a very small fraction of the sulphate was utilized. The results of this experiment, which show the utilization of sulphate as well as of other compounds, are shown in Fig. 1.

(b) The above experiment was repeated, but in order to increase the proportion of sulphur which

would be utilized the initial sulphur content of the medium was decreased to 0.35 mg/ml. A small quantity of Na_2SO_4 supplied the sulphur and 0.5% NH_4NO_3 the nitrogen.

The results of the experiment are shown in Fig. 2.

About 0.12 mg/ml of sulphate was reduced, of which 0.09 mg/ml appeared in the mycelium and 0.004 mg/ml as penicillin. No cystine or cysteine could be detected in the liquor.

Influence of nitrate and ammonia on sulphate reduction

SB medium was used in this experiment with the addition of 0.35 mg/ml of sulphur, as sulphate. Three different nitrogen sources were used: (A) 0.3% NH_3 , (B) 0.3% NH_3 plus 0.25% NaNO_3 , and (C) 0.08% NH_3 plus 1.0% NaNO_3 . Although set (A) produced more mycelium as measured by dry weight, it contained only the same quantity of mycelial total sulphur as did the others. Maximal penicillin yields were (A), 30 units/ml (day 8), (B), 29 units/ml (day 6), (C), 100 units/ml (day 16). Set B gave 53 units/ml on day 8.

Media containing cystine with different additional sulphur and nitrogen sources

(a) Three different media were made up using *SB* medium containing 0.5% NH_4NO_3 . To this were added (D) c 0.4% cysteine hydrochloride,

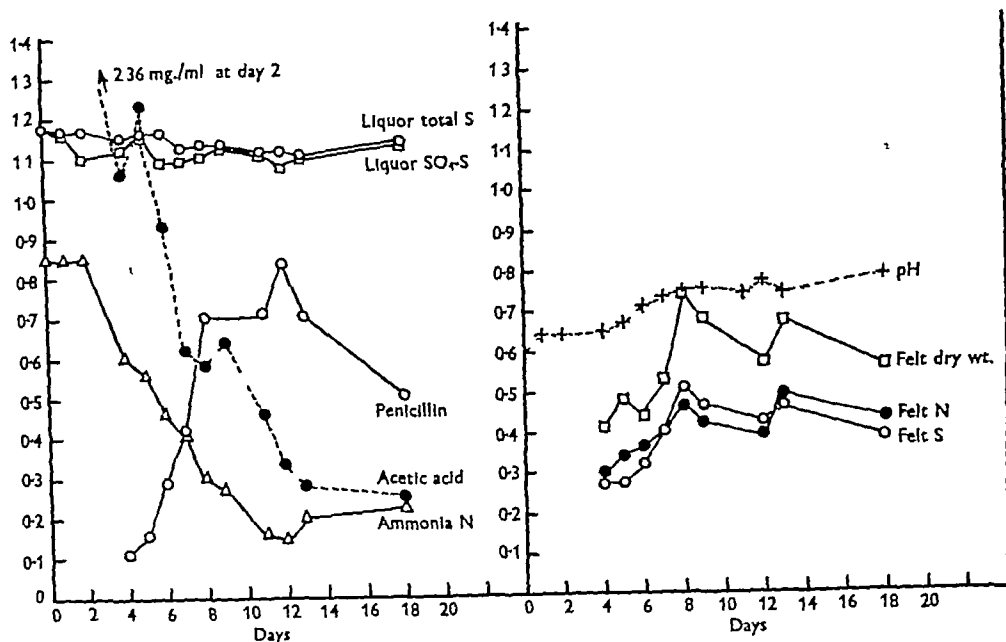


Fig 1 Sulphate metabolism of *Penicillium notatum* in relation to that of other medium components Left-hand graph \circ , liquor total S (mg/ml), \square , liquor $\text{SO}_4\text{-S}$ (mg/ml), \circ , penicillin (units/10 μl), \bullet , acetic acid (mg/ml), Δ , ammonia N (mg/ml) Right-hand graph \bullet , mycelial N (mg/ml), \circ , mycelial S (mg/10 ml), \square , mycelial dry weight (mg/100 μl), +, pH (units -10)

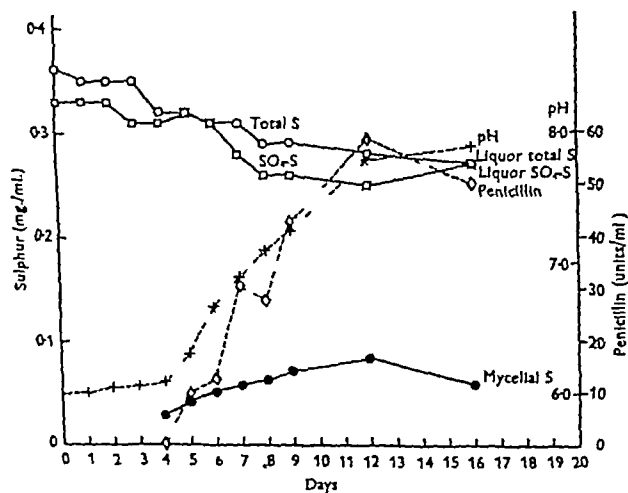


Fig 2

Fig 2 Sulphur metabolism of *Penicillium notatum* Medium $\text{SB} + \text{NH}_4\text{NO}_3 + \text{Na}_2\text{SO}_4$ \circ , liquor total S (mg/ml), \square , liquor $\text{SO}_4\text{-S}$ (mg/ml), \diamond , penicillin (units/ml) \bullet , mycelial total S (mg/ml), +, pH

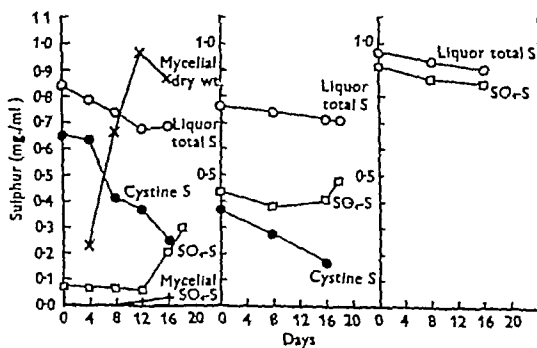


Fig 3

Fig 3 The utilization of cystine and sulphate by *Penicillium notatum* showing the conversion of part of the cystine supplied into sulphate Basal medium SB medium + 0.5% NH_4NO_3 Sulphur source 0.4% cystine (left-hand figure), 0.2% cystine + 0.2% Na_2SO_4 (centre figure) or 0.4% Na_2SO_4 only (right-hand figure) \circ , liquor total S (mg/ml), \square , liquor $\text{SO}_4\text{-S}$ (mg/ml), \bullet , cystine S (mg/ml), +, mycelial $\text{SO}_4\text{-S}$ (mg/ml), x, mycelial dry weight (mg/100 μl)

(E) c 0.2% cysteine hydrochloride plus 0.2% Na_2SO_4 , (F) 0.4% Na_2SO_4

After autoclaving the medium all the cysteine was found to have been converted to cystine and was estimated as such. As would be expected set F behaved as in the previous experiment.

In both D and E cystine fell steadily throughout. Total sulphur disappeared from the liquor until day 8 but subsequently rose again (Fig. 3). The conversion of organic sulphur to sulphate was first apparent on day 16 in both sets D and E. About 30% of the total sulphur in set D appeared as sulphate. About 15% of cystine in set E appeared as sulphate. About 5% of the total sulphur in D was detected as 'bound' (acid hydrolyzable) sulphate in the mycelium.

Penicillin yields were high in all three sets and peak titres (12 or 16 days) were 102, 117 and 92 units/ml respectively.

(b) A second experiment was carried out using L cystine (c 0.25%) as sulphur source together with (G) 0.25% NaNO_3 and 0.3% NH_3 or (H) 1% NaNO_3 and 0.25% NH_4NO_3 . The concentration of sulphur in the mycelium was the same with both media.

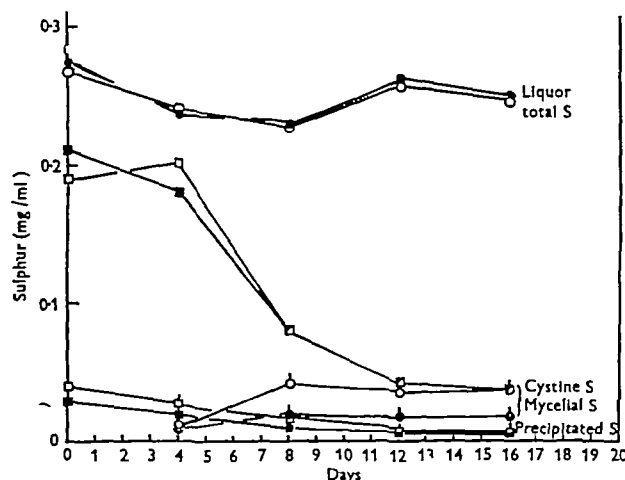


Fig. 4. The effect of nitrogen source on the utilization of cystine by *Penicillium notatum*. Medium SB containing (G) 0.25% NaNO_3 + 0.3% NH_3 , and (H) 1.0% NaNO_3 + 0.25% NH_4NO_3 . Cystine (c 0.25%) as S source. Liquor total S (mg/ml) in G, \circ , in H, \bullet . Cystine S (mg/ml) in G, \square , in H, \blacksquare . Mycelial S (mg/ml) in G, \circ , in H, \bullet . Precipitated S (mg/ml) in G, \square , in H, \blacksquare .

but transference was greater with (G) because the mycelium was heavier (Fig. 4). No sulphate could be detected in the medium in either case. Penicillin yields were low with both media (maxima 32 and 42 units/ml respectively) possibly because the concentrations of ammonia supplied were unsuitable for this purpose.

Utilization of sulphur in enriched media

In this experiment two media were used which gave thicker growth of the mould and relatively higher yields of penicillin than did SB medium. The first of these (A) containing lactose and corn steep liquor is similar to that used previously by Hockenhull (1946). This medium contains no sulphate. The results are similar to those obtained before. The second medium (B) consisted of the SB medium with the addition of 1.5% soluble starch, 0.6% NH_4NO_3 , 0.24% Na_2SO_4 and 0.3% monoethylamine. The result with this medium is shown in Fig. 5 which shows that 40% of the sulphate was

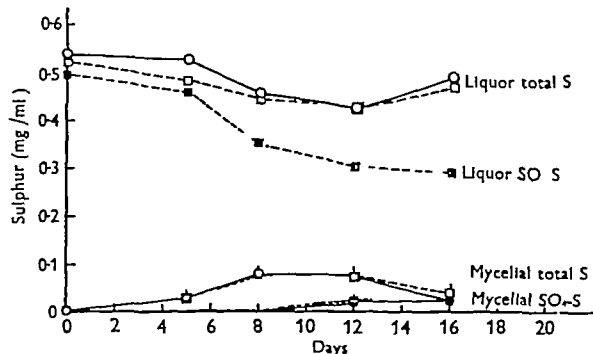


Fig. 5. Sulphate metabolism in special media. Medium A contained lactose and corn steep liquor as used by Hockenhull (1946). Medium B SB + 1.5% soluble starch, 0.6% NH_4NO_3 , 0.24% Na_2SO_4 , 0.3% monoethylamine. Liquor total S (mg/ml) in A, \circ , in B, \square . Liquor SO_4S (mg/ml) in A, \bullet , in B, \blacksquare . Mycelial total S (mg/ml) in A, \circ , in B, \square . Mycelial SO_4S (mg/ml) in A, \bullet , in B, \blacksquare . No liquor SO_4S was detectable in series A.

reduced to a soluble sulphur fraction which was not identified. Although some sulphur reappeared in the liquor from the felt, this was not in the form of sulphate. The results of this experiment are shown in Table 2.

Table 2. Development of *Penicillium notatum* on enriched media

(Media as legend to Fig. 5)

Media	Penicillin (units/ml)		Mycelial dry weight (mg/ml)		Mycelial N (mg/ml)	
	A	B	A	B	A	B
Day 8	107	86	12.0	11.3	0.85	0.88
12	107	112	14.6	11.4	0.94	0.83
16	70	80	9.7	8.0	0.55	0.52

(3) Metabolism of sulphur with simpler media

A number of media were used which were similar to, but not quite identical with, those used by Rippel & Behr (1936) and Möthes (1938) for metabolic work with *Aspergillus niger*

Sucrose medium with ammonia or nitrate

The basal medium contained 5% sucrose, mineral salts and 0.5% Na_2SO_4 . Nitrogen sources were either 0.3% NH_3 or 1.3% NaNO_3 . The former gave much heavier felts than the latter, and on corresponding days gave higher mycelial sulphur and sulphate than the other. Changes in sulphate and total sulphur in the medium corresponded with these figures, but were only a small fraction of the total sulphur available (Fig. 6)

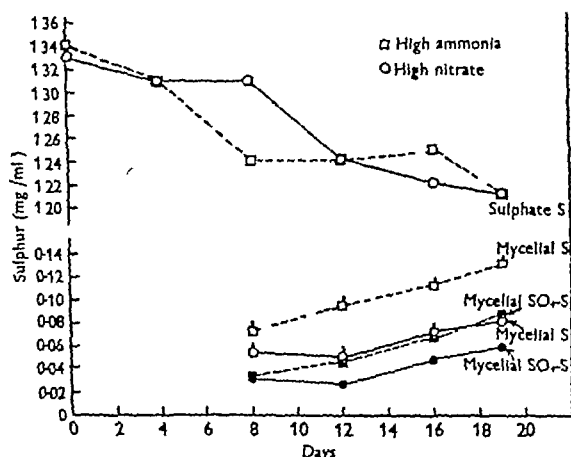


Fig. 6 Sulphate metabolism on simpler media. Basal medium 5% sucrose, mineral salts, and 0.5% Na_2SO_4 . Nitrogen source either 0.3% NH_3 or 1.3% NaNO_3 . Liquor sulphate S (mg/ml) in medium containing ammonia, \square , nitrate, \circ . Mycelial $\text{SO}_4\text{-S}$ (mg/ml) in medium containing ammonia, \blacksquare , nitrate, \bullet . Mycelial total S (mg/ml) in medium containing ammonia, \square , nitrate, \circ .

These results indicate that *Penicillium notatum* behaves quite differently from *Aspergillus niger* in that there was no tendency for organic sulphur compounds to accumulate in the medium.

Sucrose medium with urea and cystine

A medium was prepared containing 1% sucrose, 0.5% urea, mineral salts and 0.2% cystine, and inoculated with *Penicillium notatum* 1249 B21. Up to and including the twenty-third day of fermentation neither sulphate nor penicillin could be detected in the medium.

DISCUSSION

The results with *P. notatum* on the availability of various forms of sulphur are in almost complete agreement with those described by Steinberg (1936, 1941) for *Aspergillus niger*. Steinberg found that sulphate, sulphite, hyposulphite, thiosulphate and sulphamate gave maximal growth of *A. niger*, while freshly prepared sulphide or disulphide gave poor growth. Further work with organic compounds showed that L-cysteine or methionine and methoxysulphonic or *n*-butylsulphonic acids gave good growth, while mercaptans and disulphides did not. Steinberg also found that sulphate was put out of action by the addition of excess barium salt to the medium, but none of the reduced sulphur compounds was affected in this way. From these results Steinberg concluded that sulphate is not the substance through which sulphur is incorporated into the mould, but that the substance concerned is more oxidized than the sulphides. Steinberg did not consider that this compound could be H_2SO_5 in view of the stability of the S—C link in sulphonc acids (ignoring ketone bisulphite type compounds). He therefore thought it likely that the lower thio acids played the intermediate roles in the process of sulphur assimilation.

The present work, especially with the mutant strains, appears to bear out this hypothesis, for neither of these strains gave good growth with sulphate or sulphite, whereas hyposulphite and thiosulphate were utilized readily. It would therefore appear that in each of the mutants one of the links in the chain of reactions involved in sulphur metabolism is broken, and that the chain involves the reduction of sulphate to thio acids before conversion to organic sulphur compounds which are built into the mycelium. The nature of this symbiosis is as yet not fully understood. If the results so far obtained can be confirmed by a fuller investigation they should be of considerable interest. The number of steps in this process is unknown, but it would appear that the breaks occur at different places in the two mutants for they are able to grow symbiotically on sulphate. Examples of the breaking of links in metabolic chains by mutation are provided by the classical work which has been done with *Neurospora* on the formation of tryptophan and other compounds (cf. Beadle, 1945). Of more particular interest is the recent work of Lampen, Roepke & Jones (1947), in which they report the isolation of two mutant strains of *Escherichia coli* unable to utilize sulphate as sole source of sulphur but able to grow in presence of sulphite as sulphur source. One strain able to utilize sulphide only and several strains unable to use either are also reported. Similar 'parathiotropic' mutants of *Ophiostoma multianellatum* have been obtained by Fries (1945). The author

(Hockenhull, 1948) also reports mustard gas mutants of *Aspergillus nidulans* which behave in a similar manner

The metabolic experiments show that when sulphur is utilized by *Penicillium notatum* in simple media, it is nearly all transferred directly to the mycelium, and that there is little tendency for the new sulphur compounds to accumulate in the medium. When sulphur was supplied as sulphate, cystine was rarely formed in the medium, with cystine, sulphate did not usually appear in the medium. In this the mould differs from *Aspergillus niger* which has been found to produce organic sulphur from sulphate and sulphate from cystine. The metabolism of sulphur by *A. niger* is also influenced by the nature of the nitrogen source, but this effect does not seem to be so marked with *Penicillium notatum*.

With more complex media, such as those containing organic acids, there was some production of sulphate from cystine in the later stages, perhaps due to autolysis of the mycelium, but there was no accumulation of organic sulphur from sulphate. With the richest media used, containing corn steep liquor or organic acids together with starch and ethylamine as supplementary sources of carbon and nitrogen there was a marked tendency for sulphur compounds, possibly organic, to appear in the medium. These media also gave the highest yields of penicillin. Evidence that sulphate may arise from autolysis of the mycelium is provided by the fact that as much as 40% of the total mycelial sulphur was in the form of ethereal sulphate. This finding is in agreement with that of Woolley & Peterson (1937) who isolated 'cyclic' choline sulphate from the mycelium of *Aspergillus sydowii*. This sulphate may act as a reservoir of sulphur for the organism. The work with the mutants also supports the view that sulphate is not likely to be an intermediate in the metabolism of cystine. These results contrast with those obtained with *A. niger*, and also indicate that the composition of the medium has a considerable influence on the metabolism of sulphur by the organism.

Our experiments do not throw any particular light on the mechanism of the formation of penicillin.

This is not altogether surprising, since the quantity of penicillin produced is so small that the amount of sulphur involved is scarcely measurable. Since, however, considerable yields of penicillin can be obtained when other sulphur compounds are not accumulating in the mycelium it seems reasonable to suppose that penicillin is formed in the mycelium and subsequently split off by some enzymic process. The presence of cysteine, cystine or other specific sulphur compound does not seem to be a limiting factor in this process.

SUMMARY

1 The growth of normal and mutant strains of *Penicillium notatum* on different sources of sulphur has been studied, and the metabolism of some of them followed analytically. Normal strains can utilize many inorganic substances, such as sulphate, sulphite, hyposulphite and thiosulphate, as well as other compounds, and high yields of penicillin and mycelium can be obtained with a considerable variety of sulphur sources.

2 Two mutant strains which were unable to grow on sulphate, 'cyclic' choline sulphate, sodium acetone bisulphite, sulphite, or sulphamate grow well on other sulphur compounds such as cystine, thiosulphate or hyposulphite.

3 When mixed the two mutant strains could be grown together on a medium containing sulphate as sole source of sulphur. Isolates from the mixed colonies produced 'sectorized' colonies on malt medium, and from the sectors strains similar to the originals were isolated.

4 With the normal strains the utilization of sulphur, through the reduction of sulphate or disappearance of cystine, is mainly linked with the formation of mycelium. Only with certain media do non sulphate compounds accumulate in the medium. Ethereal sulphate was detected in the mycelium.

5 The metabolic route adopted for sulphur by *P. notatum* appears to be similar to that used by *Aspergillus niger*, namely that inorganic sulphur compounds are incorporated into the molecule via a chain of reduced thio acids. On the other hand, the two moulds react differently to the presence of ammonia or nitrate in the medium.

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Studies in Vitamin A

7 CAROTENE METABOLISM IN HERBIVORES

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It is generally agreed that the vitamin A found in the livers of herbivorous animals has its origin in the biologically active carotenoids present in the diet, for the intake of preformed vitamin A is nil. It has been widely assumed that the site of the conversion of the active carotenoids into vitamin A is the liver itself, although the results of *in vitro* experiments designed to test this view are equivocal (see, e.g., Woolf & Moore, 1932). In the cow, carotenoids are present in the systemic blood plasma in amounts which reflect the dietary intake of carotenoids (Mitchell & Wise, 1944), and the plasma levels presumably represent a balance between absorption from the intestine and removal by the liver, the pigments being merely *en route* to the liver from the gut. In other herbivorous animals (sheep, goat, rabbit), however, the systemic plasma contains only very small traces of carotenoids (c. 2 $\mu\text{g}/100\text{ ml}$), although considerable amounts of vitamin A are found in the liver. In all these animals the administration of vitamin A causes an increase of vitamin A in the systemic blood but administration of carotene is without apparent effect on either the vitamin A or carotene levels.

This fundamental difference between cattle and other herbivora is also shown in other ways, viz. the body and milk fat of the former contains carotenoids and is yellow, whereas that of the latter is colourless. The explanation for this striking difference between the cow and the sheep, goat and rabbit, might well be found by considering the possible factors concerned in the mode of absorption and transport of carotene from the intestine, regarding which there is at present only fragmentary experimental evidence.

Assuming that in these animals carotene* is brought to the liver in the portal blood stream and converted there into vitamin A, it follows that its presence in or absence from the systemic blood will depend on the rates of intestinal absorption and uptake (and conversion) from the portal blood by the liver. Carotene may be present in cow plasma because the rate of absorption relative to that of

uptake by the liver is high, whereas in sheep, goats and rabbits it is low and thus no carotene passes from the liver into the systemic circulation. On the other hand, if carotene is transported by the lymphatic route to the systemic circulation, so bypassing the liver, its accumulation in the systemic blood would depend on (a) the rate of absorption from the gut, (b) the rate of lymphatic transport (concn \times rate of flow), and (c) the rate of uptake by the liver.

In attempting to decide which route is concerned an obvious preliminary is to ascertain whether carotene is present in the portal blood stream after its administration in large amounts. Two difficulties arise in planning such an experimental approach. One is that absorption processes in general, and particularly those concerning fats, are retarded by anaesthesia and trauma (e.g. opening of abdomen) of several hours duration. Consequently, samples of body fluids obtained under such conditions of an acute experiment on an anaesthetized animal are much less likely to give positive results and the duration of the experiment must be considerably shorter than if the animal were conscious and unharmed. The other difficulty is that, owing to the large size and complicated nature of the ruminant stomach, the passage of material through it takes days, so that orally administered solutions are likely to enter the small intestine greatly diluted and at a very slow rate.

Both difficulties can be overcome by providing the animals beforehand at an aseptic operation with (a) a London cannula (London, 1928) on the portal vein, and (b) fistulae of either the abomasum or duodenum, so that serial portal (and systemic) samples can be withdrawn and solutions introduced directly into the intestine without operative interference.

Using these techniques, a study has been made of the carotene and vitamin A levels of portal and systemic venous blood plasma in conscious goats and sheep after the administration of large amounts of vitamin A or carotene. Consistent failure to detect carotene in transport in these experiments, and the results of concurrent investigations carried out in this laboratory (Glover, Goodwin & Morton, 1947a, b,

* Throughout this report the term 'carotene' indicates a mixture of α and β carotenes, in which the β isomer predominates.

1948*a*, *b*) compelled the conclusion that the older views on the transformation of carotene into vitamin A must be abandoned in favour of the view that the conversion takes place in the intestinal wall. Further experiments on unanaesthetized goats provided with fistulae of the thoracic duct proved this conversion to occur in the conscious animal and showed that part, at least, of the vitamin A formed is transported from the gut via the lymphatic system.

A preliminary report of the first part of this work has already appeared (Goodwin, Dewar & Gregory, 1946).

EXPERIMENTAL

Determination of vitamin A and carotenoids in blood plasma and lymph. The method for blood plasma, which was found applicable to lymph, was that normally used in this laboratory and described in detail by Glover, Goodwin & Morton (1947*c*). All optical measurements, except those in a few preliminary experiments, were carried out using the Beckman quartz photoelectric spectrophotometer. Vitamin A levels were measured by the intensity of the blue colour produced in chloroform solution at 617 $m\mu$ with the $SbCl_3$ reagent, and carotenoid levels by the intensity of direct light absorption at 449 $m\mu$ of the blood lipid extract dissolved in light petroleum (b.p. 40–60°). The standard error of a vitamin A determination is about 4%, and of a carotenoid determination 1%.

Determination of faecal carotenoids. The carotenoids were extracted according to the method of Goodwin & Morton (1948) and the carotene fraction separated by chromatography on defatted bonemeal (Goodwin & Morton, 1946).

Carotene and vitamin A supplements. The vitamin A concentrates used were potent shark liver oils. Carotene was used in the form of crystalline β carotene, red palm oil concentrate, or homogenized spinach (Nestlé). These various preparations were dispersed in a suitable volume of water by means of a Waring Blender, using, in various experiments, lecithin, bile salts and bile to aid emulsification.

Animals. Rabbits, sheep and goats were used as experimental animals. The rabbits were maintained on a diet of Lever's cubes and fresh cabbage, the sheep and goats on hay and Bibby's cattle food (No. 1 Cakelets).

The goats and sheep were provided with London cannulae (London, 1928, Gregory, 1946), abomasal or duodenal cannulae, and fistulae of the thoracic duct, at aseptic operations performed under general anaesthesia (Gregory, 1947).

Collection of thoracic lymph in the conscious animal. The thoracic duct was exposed and drained either by tying all other branches of the jugular vein, into which the duct usually opened, and then introducing a cannula into the vein, or by passing directly into the duct a thin walled plastic tube in which the lymph did not clot for many hours. The cannula or tubing was brought out through a stab wound in the skin adjacent to the incision, and lymph collected in a small rubber bag containing a small amount of heparin. In some experiments in which cannulation of the jugular was performed, clotting was successfully avoided by subcutaneous injection of 'chlorazol sky blue' in the vicinity of the incision. The dye promptly appeared in the lymph and this was stained blue and rendered incoagulable

for many hours. In other experiments, however, this method was not successful, and as supplies of suitable plastic tubing became available to us, this was used in later experiments.

The duration of collection of lymph in experiments which were successful in that clotting was avoided, was in some cases limited by the death of the animals from an unknown cause. The usual sequence of events was that, following recovery from the anaesthetic (about 15 min. after completion of the operation), the animal remained in an apparently normal state, walking about and eating, for a period of 10–20 hr. Rapid deterioration in its general condition then became apparent, indicated chiefly by stertorous and rapid breathing, and increasing weakness of the limbs. Death from respiratory failure occurred within 1–2 hr. from the first appearance of these signs and post-mortem examination failed to reveal any condition likely to have been responsible for death. The rate of flow of lymph was constant at about 30–50 ml/hr. and tended to decrease during the period of introduction of the carotene solution into the intestine, particularly if this was rapid. The flow continued up to the moment of death. In other experiments, which were failures owing to clotting of the lymph, the animals recovered completely and regained a normal healthy state, and as in these cases the same procedures had been carried out, it seems unlikely that any of these was itself a factor in the death of the other animals.

Since deterioration in the animal's condition would be expected, if anything, to depress absorptive processes, and in the experiments reported here absorption of the solution introduced (as indicated by the presence of vitamin A and fat in the lymph samples) was, in fact, well advanced before the death of the animal, we feel that the fact that these experiments were semi-acute, in that they were not followed by permanent recovery of the animal, does not detract from their value.

RESULTS

Carotene and vitamin A in portal and systemic venous blood

A Rabbits. Experiments were first carried out to ascertain whether oral administration of massive doses of carotene was followed by the appearance in the systemic blood of appreciable amounts of carotene. Crystalline β carotene dissolved in refined arachis oil or dispersed in water was administered by stomach tube to groups of 5 rabbits in doses of 4800 μg each. In two experiments the blood samples were drawn at intervals of 3, 6, 27, 48, 72 and 96 hr. after dosing, and in a third at hourly intervals for the first 4 hr. after dosing. These experiments were carried out before the photoelectric spectrophotometer was available, and in view of the large quantities of plasma required for a determination it was considered preferable to pool the samples from the group rather than to withdraw excessive amounts of blood from any one animal. In no instance was carotene observed in the plasma. The possibility that the absorption was defective

was investigated by determination of the faecal carotene content, it was found that whereas absorption from arachis oil was poor (almost 100 % excretion) that of the colloidal solution was apparently good, only 13.6 % of the administered carotene being accounted for in the faeces. The bulk of the carotene given in this form had either been absorbed or destroyed in the intestine, experiments in which colloidal carotene solutions were incubated at 37° with rabbit intestinal contents provided no evidence for such destruction. It seemed reasonable to conclude therefore that the carotene had been absorbed without gaining access to the systemic circulation.

Examination of the portal blood for vitamin A and carotene under similar conditions was obviously indicated, and in the hope of avoiding more complicated experimental procedures, a number of acute experiments were performed on rabbits in which blood samples were taken simultaneously from the portal vein and the inferior vena cava under light nembutal or cyclopropane/O₂ anaesthesia at various times after the oral administration of large doses of carotene. No detectable amounts of carotene were found in any of the blood samples.

B Sheep and goats In the experiments just described, failure to detect carotene in the portal blood was not unexpected in view of the notorious sensitivity of absorption processes to the conditions of such experiments. Further experiments were therefore carried out on goats and sheep provided with portal and abomasal cannulae.

In these experiments, the results of three of which are summarized in Table 1, the carotene preparation (dispersed unsaponifiable matter from red palm oil prepared by the method of Goodwin & Morton, 1946) was introduced slowly into the abomasum and 20 ml samples of portal and systemic venous

blood were drawn simultaneously at frequent intervals for periods up to 24 hr after dosing. The results were consistently negative, the carotene values of the bloods were so low as to be indistinguishable from zero for they correspond to a reading on the spectrophotometer of $E=c \cdot 0.01$. The vitamin A levels were, on the whole, reasonably constant, and certainly showed no significant rise after administration of the carotene.

Two similar experiments were performed on sheep, in one of which the carotene was administered in the form of spinach purée. Both gave results identical with those obtained on goats, and are therefore not reported in detail.

Extent of absorption of carotene from the alimentary tract

A possible explanation of the negative results so far recorded was that little or none of the carotene given in the manner described had been absorbed. An experiment carried out on a sheep, in which vitamin A was administered under similar conditions instead of carotene, showed that this is well absorbed, a normal blood tolerance curve being obtained. The vitamin A levels in the portal and systemic blood samples were never significantly different, and the time required to reach maximum blood levels was 4–6 hr, approximately the same as that needed in man (Table 2).

However, as the dose of vitamin A used was considerably higher than that of carotene and as it is well known that vitamin A is more readily absorbed than carotene (e.g. Wald, Carroll & Sciarra, 1941), further experiments were made to check more directly the efficiency of carotene absorption. In goat no. 2 (Table 1) carotene was introduced in the

Table 1 *Carotene and vitamin A levels of portal and systemic blood*

(Samples of plasma of goats drawn at varying intervals after administration via an abomasal cannula of 31,000 µg carotene as red palm oil unsap. dispersed in 2 l of water with potassium stearate.)

Goat no.	Time after dosing (hr.)	Plasma carotene and vitamin A levels			
		Systemic		Portal	
		Carotene (µg/100 ml)	Vitamin A (i u/100 ml)	Carotene (µg/100 ml)	Vitamin A (i u/100 ml)
2	0	3.9	44	3.0	43
	3½	3.1	39	2.8	29
	7	2.3	—	7.2	—
	24	6.1	36	1.5	23
3	0	5.6	43	7.1	47
	4	4.2	39	6.6	66
	8	3.4	60	4.2	76
	24	1.8	50	2.4	56
4	0	4.2	49	2.4	55
	1	—	56	3.0	47
	3	1.8	30	3.0	47
	6	1.2	—	1.2	35
	24	1.8	—	1.2	32

Table 2 *Vitamin A levels of the portal and systemic blood*

(Samples from a sheep after receiving 3×10^6 i u of vitamin A dispersed in 2 l of water with 0.2% (w/v) of bile salts and 0.1% (w/v) sodium stearate)

Time after dosing (hr)	Vitamin A levels (i u/100 ml plasma)	
	Portal	Systemic
0	20	28
1½	68	73
4½	131	129
7	121	116
24	99	118
72	48	—

Table 3 *Excretion of carotene*

(Estimation of carotene in fluid draining from an ileal fistula of a sheep fed 32,850 µg of carotene as homogenized spinach via an abomasal cannula)

Time of draining after administration of carotene (hr)	Volume of liquid (ml)	Carotene content (µg)	Hourly excretion (µg)
3	50	Nil	Nil
5	20	Trace	Trace
7	Nil	Nil	Nil
11	160	427	107
14	250	58	194
16	Trace	Trace	Trace
21	250	300	60
23	50	252	126
25	50	400	200
28	50*	106*	70
32	50	252	63
34	150	163	82
37	200	840	280
49	100	1250	104
Total 4048			

* Incomplete collection about 75% of total

usual way into the abomasum and this was emptied 5 hr afterwards. Only 6% of the carotene introduced was still present, indicating that there was no appreciable retention in the abomasum. The excretion in these animals was ascertained in two ways: (a) by the usual balance experiment in which the amount of carotene excreted for several days before and after administration of a known dose was followed, and (b) by providing a sheep with a cannula in the terminal ileum in addition to that in the abomasum so that after administering carotene all material which passed through the small intestine could be collected and examined. This sheep received into the abomasum homogenized spinach containing 32,850 µg of carotene, and the ileal contents were collected for the next 48 hr (Table 3). From the results it was estimated that 87.7% of the carotene had failed to reach the terminal ileum.

The balance experiments were carried out on two goats each provided with (a) a portal cannula, and (b) an abomasal or duodenal fistula. As it was not feasible to remove hay from the diet either before or during the experiment, a base-line of carotene excretion had to be determined. This was obtained by taking a weighted mean value for the daily carotene excretion over 5–7 days previous to the experiment and for a similar period after the experiment, when the levels had returned to, or dropped below, the original levels. This tended to eliminate any error due to variation in the amount of hay consumed by the animals during the experiment. Table 4 records the results of two such experiments, which indicate that carotene is well absorbed under these conditions. Only 18.9 and 7.1% respectively of the carotene given appeared in the faeces.

It seemed probable from the results of the experiments in the previous series that the carotene was largely absorbed, but possibly at a rate which was slow and dependent on the rate of passage from the abomasum, so that detectable amounts of carotene

Table 4 *Faecal excretion of carotenoids*

(Goats fed via a duodenal cannula, red palm oil unsap dispersed in water with lecithin and bile salts)

Goat no 5 (abomasal tube)			Goat no 6 (duodenal tube)		
Duration of collection	Total daily excretion (µg)	Excess excretion over basal (µg)	Duration of collection	Total daily excretion (µg)	Excess excretion over basal (µg)
5 days prior to dosing	2,205	—	7 days prior to dosing	2,615	—
2 days after dosing	13,460	9,390	1 day after dosing	5,245	2,700
1 day later	12,970	10,935	2 days later	16,360	11,270
1 day later	3,080	1,045	5 days later	2,250	—
1 day later	1,190	—			
Weighted mean of basal excretion (µg)	2,035		Weighted mean of basal excretion (µg)	2,545	
Total excess excretion (µg)	21,370		Total excess excretion (µg)	13,970	
Dose (µg)	113,000		Dose (µg)	197,400	
Percentage excretion	$\frac{21,370}{113,000} \times 100 = 18.9$		Percentage excretion	$\frac{13,970}{197,400} \times 100 = 7.1$	

in the portal venous blood were not observed. Goats were therefore provided with portal cannulae and duodenal cannulae in the hope that direct introduction of carotene solutions into the upper parts of the small intestine would result in more rapid absorption. Larger amounts of carotene were also

Table 5 *Vitamin A plasma levels of portal and systemic blood*

(Samples drawn from goats at varying intervals after administration of 100,000 μg of carotene, via a duodenal cannula, as red palm oil unsap dispersed in 2 l of water with lecithin and bile salts)

Animal no	Dose level (μg)	Time after dosing (hr)	Plasma vitamin A levels (i u /100 ml)	
			Systemic	Portal
6	197,400	0	65	72
		4	67	75
		10	67	67
		24	64	62
7	39,600	0	90	104
		4*	99	105
8	197,400	0	86	90
		4	85	106
		8	84	105
		24	82	88

* Goat died

used. In three experiments of this type, in which samples of portal and systemic venous blood were drawn at intervals after the duodenal introduction of carotene, no detectable amounts of carotene could be observed (Table 5). The results on goat no. 6 were obtained during the balance experiments previously described.

The implications of our inability to demonstrate carotene in the blood of goats and sheep were becoming increasingly clear, the carotene was being converted into vitamin A before it reached the blood, i.e. in the intestinal wall. Concurrent experiments which were being carried out in this laboratory on rats and which are reported fully in an accompanying paper (Glover *et al.* 1948b) made it clear that this was the case. A review of all the vitamin A plasma levels obtained in our previous experiments did not reveal the expected rise following carotene administration. It was possible that the rise was so slight that it could not be detected by the analytical methods available.

It is reasonably certain that vitamin A itself travels to the liver via the lymphatic pathway (Drummond, Bell & Palmer, 1935; Popper & Volk, 1944; Eden & Sellers, 1948). To complete this investigation, therefore, it was decided to provide goats with thoracic cannulae as well as duodenal tubes so that thoracic lymph could be collected continually during the experiment. In two preliminary experiments in which the unsaponifiable fraction of red palm oil, dispersed in 1-2 l water, was administered quickly, no carotene appeared in the lymph but there were small increases in the vitamin A levels. The clearly positive results on three animals recorded in Table 6 were obtained after administering the carotene dispersed in a much smaller volume of water (250 ml) slowly over 6-8 hr.

The concentration of the lymph lipids extracted by light petroleum (b.p. 40-60°) was also determined in these experiments and the variations in it follow closely those for vitamin A. The lipid and vitamin A

Table 6 *Vitamin A levels in the thoracic lymph*

(Samples drawn at intervals after administration of 112,000 μg of carotene, as red palm oil dispersed in 250-500 ml of water with either bile or bile salts)

Time after dosing (hr)	Vitamin A lipids in lymph					
	Goat no. 12		Goat no. 13		Goat no. 14	
	Vitamin A (i u /100 ml.)	Lipids (mg /100 ml.)	Vitamin A (i u /100 ml.)	Lipids (mg /100 ml.)	Vitamin A (i u /100 ml.)	Lipids (mg /100 ml.)
0 Control	43	34	69	173	29	256
1	—	—	—	—	20	394
2	64	107	64	167	30	351
3	—	—	—	—	31	429
4	79	131	63	228	33	455
5	—	—	—	—	45	419
6	80	165	79	310	55	1390
7	—	—	—	—	54	1830
8	72	232	82	669	48	1342
9	—	—	—	—	45	1270
10	84	498	147	1053	42	1209
11	—	—	—	—	52	1428
12	77	597	97	957	45	1128
13	—	—	—	—	—	—
14	101	262	—	—	—	—
15	—	—	—	—	—	—
16	—	195	—	—	—	—
17	—	—	—	—	—	—
18	67	67	—	—	—	—

values obtained on goat no 14 are presented graphically in Fig 1. The parallelism is very striking and the drop in absorption after 6 hr when the carotene addition was discontinued and the increase after 9 hr when it was resumed, is well reflected in both curves. The increase in the lymph lipids was, however, much greater than that in the vitamin A levels. The maximum increase in vitamin A obtained in each experiment was c 100 %, whereas that of the lipids was of the order 500–800 %. The reason for this is not immediately apparent, but the well-known inhibition of the antimony trichloride test by excess fat probably makes our vitamin A values determined around maximum absorption rather low.

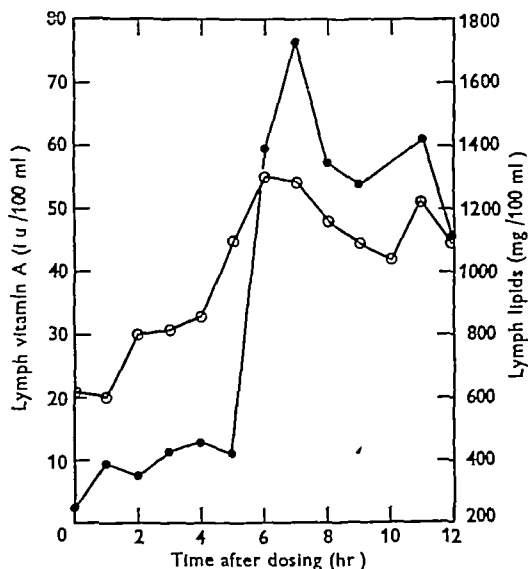


Fig 1 Variation in lipid and vitamin A levels of lymph of a goat dosed with carotene (112,000 μ g as red palm oil) via a duodenal cannula. o o o o vitamin A, • • • • lipids

Two other interesting but limited observations were made on the lymph. (a) The vitamin A levels of two samples of goat lymph were much less than the levels in the corresponding plasma. The two lymph samples contained 29 and 37 i u / 100 ml, whilst the corresponding plasma levels were 135 and 67 i u / 100 ml respectively. One experiment indicated that at least 85 % of the lymphatic vitamin A is esterified. A recent preliminary note (Eden & Sellers, 1948) states that vitamin A in the lymph of bullocks is also preponderantly ester, but that the lymph levels are higher than the plasma levels. (b) Light petroleum (b.p. 40–60°) extracts of lymph obtained from goats recently removed from summer pasture were quite yellow, although similar extracts from the corresponding blood plasma were colourless, extracts from lymph of goats kept on hay and cattle food were also colourless. Spectrophotometric examination of the pigment indicates that it is not a carotenoid, the

absorption curve (Fig 2) shows only one maximum at 433–434 $m\mu$ and a very small inflexion at about 590 $m\mu$ (not recorded in the curve). The pigment is destroyed by treatment with alkali, which further indicates that it is not a carotenoid. From these data the possibility that it is derived from chlorophyll must be considered, but further investigations are necessary finally to determine this.

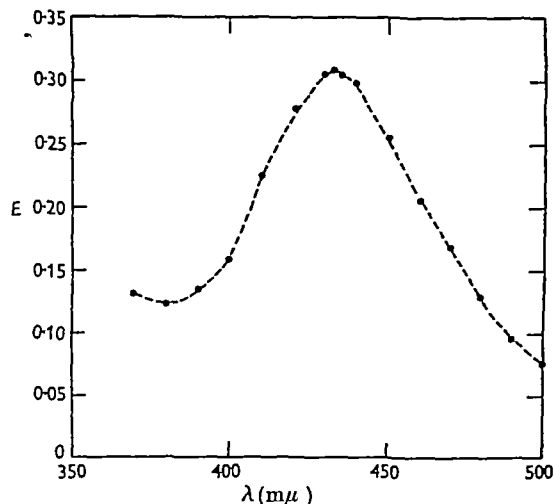


Fig 2 Absorption spectrum of a pigment extracted by light petroleum from lymph of a goat grazing on fresh pasture

Carotenoids in other tissues

The apparent selective accumulation of carotenoids often reported in certain mammalian tissue, viz adrenals, corpora lutea, yellow bone marrow, has led to a certain amount of speculation as to the function of carotene *per se*, as distinct from its activity as a vitamin A precursor. It was obviously important to determine whether, in those animals in which carotene appears to get into the blood stream only in traces if at all, carotene accumulates in these organs. The adrenals, ovaries, liver and bone marrow (from the long bones) of a number of goats killed after carotene administration were examined. In no case was carotene detected in any of the organs. The bone marrow was perfectly white.

DISCUSSION

The failure to obtain positive results in our first series of experiments, carried out to determine the route whereby carotenoids are transported in sheep and goats, is explicable, in the light of our later work. We have now shown that in the unanaesthetized animal the carotene is converted into vitamin A in the gut wall and transported via the lymph to the liver where it is stored. It is difficult in experiments of this type to rule out completely the lumen of the intestine as the site of conversion, but the

failure to destroy carotene by incubation with rabbits' intestinal contents (*vide infra*) indicates that this is so, and the experiments on rats (Glover *et al* 1948b) supply additional proof. The transport of the vitamin A via the portal system is not ruled out but is highly unlikely to occur to any great extent. This confirms the work of Drummond *et al* (1935), Popper & Volk (1944), and Eden & Sellers (1948). It is suggested that the failure to find vitamin A in the plasma of sheep and goats after feeding carotene is due to the dynamics of the situation. The vitamin A is taken up by the lymph in quantities which are sufficiently great to be experimentally demonstrable, however, in the delivery of the lymph into the systemic blood supply the excess vitamin A becomes so quickly dispersed and removed that the concentration increment is too small to be detected.

Support for this reasoning has been provided by the following experiment. 10^6 i.u. of vitamin A were administered in similar dispersions (250 ml water, 1% bile salts) via duodenal cannulae into two goats, one of which was provided with a thoracic cannula, and blood and lymph samples were collected from both animals at varying times afterwards. It is clear (Table 7) that the increase in vitamin A is much greater in lymph than in blood and obviously there is also a dilution aspect when lymph is compared with blood. Complete confirmation of this observation has been provided by the recently reported work of Eden & Sellers (1948).

Table 7 *Comparison of vitamin A levels in plasma and lymph of goats*

(The goats received identical doses of vitamin A (10^6 i.u.) via duodenal cannulae.)

Time after dosing (hr.)	Goat no 16 vitamin A (i.u./100 ml plasma)	Goat no 17 vitamin A (i.u./100 ml lymph)
0	55	33
2	—	40
3	87	—
4	—	83
6	167	189
8	181	232.5
10	—	452
12	—	517
14	—	496
16	—	293
18	—	232
24	78	—

The fact that in goats the normal levels of vitamin A in lymph are lower than the blood levels is not in opposition to this point of view. Normally, the vitamin A in blood is in the alcohol form and its concentration is controlled at an optimum functional level by the liver (Glover *et al* 1947c). The vitamin A in normal lymph is that which has been formed from

carotene and is now in transport to the blood, in other words, in a normal animal, blood and lymph vitamin A levels need not and do not bear any relationship to each other.

It is now possible to sum up the position regarding the conversion of carotene into vitamin A in mammals. Although the experimental basis for the hypothesis has been severely criticized (Rea & Drummond, 1932; Woolf & Moore, 1932), it was generally assumed until recently that the conversion takes place in the liver. Sexton, Mehl & Deuel (1946) first suggested that the liver was not the site of conversion, and demonstrated that injected carotene accumulated in the liver but did not appear to be converted into vitamin A. The first results reported here (see Goodwin *et al* 1946) also pointed in this direction, and when Glover *et al* (1947b, 1948a) found that retinene, a possible intermediate in the carotene vitamin A transformation, was converted into vitamin A in the gut, it appeared even more probable that conversion of carotene takes place in this organ. The accompanying paper (Glover *et al* 1948b) proves this for the rat by experiments carried out *in vitro* and *in vivo*, and Wiese, Mehl & Deuel (1947) and Mattson, Mehl & Deuel (1947) have completely confirmed these results. Meanwhile, Thompson, Ganguly & Kon (1947), approaching the problem from a slightly different angle, have come to the same conclusion, using the pig. The work reported here on goats confirms these findings, and proves that the vitamin A thus formed is transported via the lymphatics, a fact previously suggested in an acute experiment by Thompson *et al* (1947).

SUMMARY

1 Carotene could not be demonstrated in the portal or systemic blood of rabbits, goats or sheep after administration of massive doses of carotene in various forms by the oral, abomasal or duodenal route.

2 Carotene, either as dispersed red palm oil or homogenized spinach, is well absorbed by goats and sheep irrespective of its route of administration.

3 In goats provided with thoracic cannulae, increases in the vitamin A levels of the lymph were obtained after feeding carotene, proving that vitamin A is formed from carotene in the gut wall and transported to the liver via the lymph.

4 Increases in vitamin A plasma levels, to be expected if carotene is converted into vitamin A in the gut wall, could not be demonstrated. The probable reason for this is discussed.

5 After a dose of vitamin A the concentration of vitamin A was much higher in lymph than in plasma.

6 A restricted number of experiments indicate that the normal vitamin A level in the lymph in

goats is less than that in the plasma, and that the lymph vitamin A is mostly in the ester form

7 A non carotenoid yellow pigment, which may be a chlorophyll derivative, is present in the lymph of goats feeding on fresh grass

8 No carotene could be detected in the adrenals, liver, ovaries, or bone marrow of goats

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Studies in Vitamin A

8 CONVERSION OF β CAROTENE INTO VITAMIN A IN THE INTESTINE OF THE RAT

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Since the work of Moore (1930), which showed that carotene administered orally to the rat was converted into vitamin A, which was stored in the liver, many investigators have attempted to determine the locus of the transformation. The problem has been approached in different ways and attention has been focused on two organs, the intestine and the liver, in either of which 'carotene'* given by the oral route could conceivably be transformed into vitamin A.

The results of the earlier *in vitro* work, where minced liver tissue or extracts were incubated with

* 'Carotene' is used here to denote mixtures of the α and β isomerides

colloidal suspensions of 'carotene', were conflicting. Ahmad (1931), Olcott & McCann (1931), Parenti & Ralli (1932) and von Euler & Klusmann (1932) obtained evidence for the formation of vitamin A as indicated by the production of a lipid with an ultra-violet absorption band in the region of 328 m μ (the maximum for vitamin A) or by the detection of an increase in colour intensity with the antimony trichloride reaction. On the other hand, Rea & Drummond (1932), von Euler (1932) and Ahmad (1934), were unable to prepare vitamin A under similar conditions. Furthermore, Drummond & MacWalter (1933) could not obtain vitamin A from carotene which was actually taken up by the liver cells prior

to mincing and incubating. In all the experiments for which positive results were claimed, at most only traces of vitamin A appear to have been formed. Again, it is now well known that under the action of heat β carotene yields *cis* isomerides, solutions of which in saturated hydrocarbon solvents exhibit an absorption maximum *c* 335 $m\mu$, which is difficult to distinguish from the vitamin A maximum when other absorbing substances are present. Hence the results of *in vitro* experiments are not considered unequivocal proof that the conversion takes place in the liver.

The *in vitro* technique having failed to solve the problem, workers turned their attention to studies on the living animal. If carotene administered by a parenteral route shows vitamin A activity, then the liver is the probable site of conversion of the provitamin. Carotene in colloidal suspension has been administered parenterally by various groups of workers and again the results were conflicting or indefinite. Using the intravenous route, Wolff, Overhof & van Eekelen (1930) and Ahmad, Grewal & Malik (1934), obtained positive results for the rabbit, but Rea & Drummond (1932) and Drummond & MacWalter (1933) could not confirm the transformation of carotene to vitamin A in the liver of the cat or the rat.

More recently, using a technique of partial hepatectomy similar to that of Drummond & MacWalter (1935), Vmet, Plessier & Raoul (1943) claim to have demonstrated the conversion of carotene to vitamin A in the liver of the rabbit. The analysis of small specimens of this tissue taken at intervals after injection of a colloidal suspension of carotene showed an increase in vitamin A in less than 15 min., whereas controls showed a drop in vitamin A content in that period. The carotene content of the specimens was determined at the same time. No values greater than 2.16 μg were recorded, suggesting that an immediate conversion of the carotene had occurred on reaching the liver. This observation is in conflict with the fact that the transformation of carotene into vitamin A is a comparatively slow process, and also with the observations of van den Bergh, Muller & Brockmeyer (1920), Drummond, Gilding & MacWalter (1934) and many others, that carotene introduced into the circulation intravenously is stored in the liver, particularly in the Kupffer cells. So, apart from the difficulties of partial hepatectomy (both as to technique and interpretation) emphasized by Drummond & MacWalter (1935), the evidence of Vmet *et al.* is not satisfactory in establishing the liver as the site of conversion.

Using curative or growth tests on vitamin A-depleted animals as the criterion of activity, Chu & Coady (1934), With & Wanscher (1939) and Tomarelli, Charney & Bernhart (1946) have claimed that carotene possesses vitamin A activity when

administered by the intramuscular route. Yet Rokhlina, Balakhovskii & Bodrova (1943) came to the conclusion that carotene administered parenterally is completely lacking in vitamin A effect, regardless of whether an oil or an aqueous colloidal solution is used. Again, the evidence is conflicting.

Recently, Sexton, Mehl & Deuel (1946) have carefully compared the biological activity of carotene administered to the rat orally and parenterally. When carotene was given by the parenteral route moderate amounts were stored in the liver but only traces of vitamin A were found there. When given by the oral route, however, in smaller doses spread over a period, moderate amounts of vitamin A, but little carotene, appeared in the liver. Furthermore, vitamin A deficient rats were unable to utilize carotene given by intrasplenic injection, for the symptoms of vitamin A deficiency persisted, although substantial amounts of carotene had reached the rats' livers. The authors suggested that this negative evidence excluded the liver and pointed to the intestine as the site of conversion.

It appears from these results that the biological activity of carotene is at best very much less when given by the parenteral route than when fed orally, while direct analysis of the liver tissue shows that substantial amounts of β carotene may be stored there (in spite of the fact that the rat is an efficient converter of carotene to vitamin A) and yet not be utilized by the animal to alleviate symptoms of vitamin A deficiency. Thus, even if the liver can produce traces of vitamin A from the provitamin, it can be neither the main nor the only locus of the transformation.

The next organ to consider as the site of conversion is the intestine, which has not hitherto been examined so extensively. Moore (1931) carried out various colorimetric tests on intestinal lipids. He used a ratio expressing the intensity of light absorption due to visible pigment relative to that of the blue colour produced with the antimony trichloride reagent to detect the presence of vitamin A. The observed change in the ratio (a fall) was not, however, sufficient to establish the presence of vitamin A. Using the same criterion for vitamin A, Vmet *et al.* (1943) examined the intestinal contents and mucosa of vitamin A-depleted guinea pigs 2-6 hr after oral administration of carotene, and obtained a similarly indefinite result. Sexton *et al.* (1946) dosed vitamin A depleted rats with carotene and then removed the intestinal tracts and incubated them for 6-24 hr at 37°. No evidence was obtained either of conversion of carotene to vitamin A or of its destruction.

Popper (1941) studied the problem using the method of fluorescence microscopy. By examining the various organs of a series of vitamin A depleted rats at intervals after administering carotene orally

and parenterally, he attempted to find the organ in which fluorescence first appeared. He never detected fluorescence due to vitamin A in the liver of the rat following parenteral administration of carotene, after oral administration he sometimes detected fluorescence first in the intestine and later in the liver, but usually first in the latter. Since he was occasionally able to detect vitamin A fluorescence first in the intestine Popper suggested that this organ could convert carotene into vitamin A, though he believed the liver to be the main site of conversion.

It appears likely that the conversion can take place in the intestine, but the fact that the reaction is slow probably accounts for the lack of absolute proof. The mechanism of the transformation reaction is not known. Hunter (1946), following the work of Morton & Goodwin (1944) on retinene, suggested that oxidative attack upon the central double bond occurs resulting (possibly with intermediate stages) in formation of vitamin A aldehyde, which in turn is reduced to vitamin A alcohol. He has shown that such a scheme can be realized *in vitro*, albeit with very small yields. Glover, Goodwin & Morton (1948) have shown that retinene₁ (vitamin A aldehyde) fed to rats is reduced immediately on entering the intestinal wall. This strengthens the hypothesis that the aldehyde is a link in the conversion of β carotene to vitamin A in the intestine.

In the light of the foregoing considerations, the aim of the present work was to examine more closely the possibility that the intestinal wall is the site of the transformation. A preliminary report of the work has already been published (Glover, Goodwin & Morton, 1947*a*). The following possible reactions were considered: (a) breakdown of β carotene to vitamin A aldehyde, (b) reduction of the aldehyde to the alcohol, and (c) absorption and esterification of vitamin A alcohol followed by its removal from the intestine into the blood stream. If (b) and (c) take place more rapidly than (a), no appreciable amount of vitamin A will be found in the intestine even at the height of absorption of β carotene. Compared with vitamin A, carotene is poorly absorbed even under the most favourable circumstances. The results on the absorption of vitamin A aldehyde (Glover *et al.* 1948) indicate that processes (b) and (c) are very rapid indeed, but little is known about (a). With (1941) compared the rate of deposition of vitamin A in the liver after feeding carotene and vitamin A. He found that vitamin A appeared in the liver not earlier than 2 hr after ingestion of carotene, whereas only 0.5 hr was required for the preformed vitamin to reach that organ. This suggests a moderate time lag for process (a). Rea & Drummond (1932) and Drummond & MacWalter (1933) have come to a similar conclusion. Furthermore, many workers have been unable to

observe any appreciable rise in the plasma level of carotene or vitamin A in human subjects given moderate doses of carotene, e.g. von Drigalski (1934), With (1941), and Steigmann & Popper (1944). Similarly, Goodwin, Dewar & Gregory (1946) and Goodwin & Gregory (1948) could not detect a rise in carotene or vitamin A in portal or systemic blood when large doses of carotene were fed to goats through a duodenal fistula. The above observations can only be satisfactorily explained if process (a) were much slower than (b) or (c). Hence one cannot expect to find much vitamin A in the rat intestinal wall unless a moderately high concentration of carotene can be obtained there. It is hoped to revert to the dynamics of the processes at a later date.

EXPERIMENTAL

Preparation of carotene. In the preliminary experiments, the carotene used was prepared from dried grass, which was extracted with acetone in a large Soxhlet extractor. The solvent was removed from the extract and the residue, taken up in light petroleum, was chromatographed on a column of alumina (Savory and Moore) to remove chlorophylls and xanthophylls. The light petroleum eluate was made up to a known volume and the carotene content of the solution determined by measuring its extinction at 448 m μ , using the Beckman quartz photoelectric spectro-photometer.

To a known volume of this solution a measured quantity of arachis oil was added and the solvent removed, so that the desired amount of carotene was contained in 1 ml of the oil. For the later more quantitative experiments, in which the absorption spectra of the tissue lipids were recorded, crystalline β carotene was used. The requisite amount was dissolved in diethyl ether (freshly distilled over reduced Fe) and again a quantity of arachis oil containing a little lecithin (0.1 g/ml) was added and the solvent removed *in vacuo* at room temperature leaving a solution of β carotene of known concentration in the oil (2–5 mg/ml). Tocopherols or other anti-oxidants were not added to this solution because their presence would complicate the ultra-violet absorption spectra of the lipid extracts from the various tissues.

Animals. Two groups of adult rats (5 males and 5 females) were prepared as controls by feeding them on the normal diet of cubes (Lever Bros. and Unilever Ltd.) supplemented with carrots and cabbage for approximately 3 months. On this diet the rats build up a moderate liver reserve of vitamin A solely from a provitamin source, as the cubes when used contain no detectable vitamin A but a certain amount of carotene (c. 5 μ g/g). At the end of this period the rats were taken off the carotene diet and placed on a vitamin A free diet (Glover, Goodwin & Morton, 1947*b*) for 4 days to allow the residual carotene in the intestine to disappear. They were then killed and their livers and small intestines removed for examination.

Leaving out preliminary experiments, a further group of 28 newly weaned rats were placed on a vitamin A free diet for about 5 weeks, by which time growth had ceased. A group of 4 were killed as controls. The pooled livers and pooled intestines were separately saponified. Each

non saponifiable extract was tested for vitamin A with SbCl_3 . The negative results obtained confirmed the absence of detectable amounts of vitamin A.

Except for 3 which were examined singly at higher dose levels, the remainder of the rats were divided into groups of 3-5. The animals were fasted for 15 hr prior to feeding β carotene. The solution of β carotene in oil (2-5 mg/ml) was taken up in a syringe and 1 ml administered by stomach tube to each animal in the group. For the single animals fed at higher dose levels (one at 10 mg and two at 14 mg each) the β carotene was contained in 2 ml oil. A little of the β carotene in the oil used at the highest dose level did not appear to be in true solution.

Preparation of tissue extracts and analytical methods

At various intervals (5-24 hr) after administering the β carotene, the animals were anaesthetized and killed. The liver and intestinal tract, excluding the large intestine, were removed for examination. The former was ground with anhydrous Na_2SO_4 and extracted with freshly distilled ether. The extract was then examined for vitamin A and carotene by the methods to be described. The small intestine was separated from the stomach at the pyloric sphincter. The stomach was cut open and the contents removed with a little ethanol and ether. The mixture was transferred to a separatory funnel and after the addition of a suitable quantity of water, extracted with ether. This extract was washed with water and dried over Na_2SO_4 . The solvent was then removed, and the residue dissolved in light petroleum and chromatographed on bone meal to remove the residual β carotene. The adsorbate was removed with acetone and the lipid residue was recovered and examined for vitamin A.

The contents of the small intestine were flushed out with 0.9% NaCl and the mixture collected in a beaker. Approximately 0.5 vol of ethanol was added and the whole triturated in a mortar to break up any solid material. The mixture was then transferred to a separating funnel and extracted with ether until free from colour, the ether extract being then treated as described for the stomach extract. The intestinal tissue was saponified and extracted with ether. The washed extract was dried over Na_2SO_4 , filtered and the solvent removed, the residue was taken up in 20 ml light petroleum and chromatographed on a bone meal column. The light petroleum eluate was made up to a known volume, usually 100 ml. The adsorbate was eluted with acetone, the solvent removed and the lipid residue taken up in 10 ml light petroleum or methanol in a 25 ml flask. The flask was placed in a previously prepared ethanol CO_2 mixture at -80° for 1 hr, during which time a large proportion of the sterols was precipitated. This was filtered off on a small G 4 sintered glass funnel containing and surrounded by pieces of solid CO_2 . The clear filtrate was collected in a small filter tube. Suitable samples were taken for determination of the ultraviolet absorption spectrum and of vitamin A.

Incubation of intestines Rats were anaesthetized 8 hr after being dosed with carotene and the small intestines ligatured at the pyloric sphincter and at the caecum. The closed intestines were then immediately removed and placed in Ringer solution and incubated at 37° . At the end of the required period (3-20 hr) they were removed and treated as described for the normal intestine.

Determination of carotene and vitamin A The solutions of carotene were examined in the Beckman quartz photoelectric spectrophotometer. The extinction of the solutions was measured at 448-450 $\text{m}\mu$, and accepting the $E_{1\%}^{1\text{cm}}$ (450 $\text{m}\mu$) for β carotene in light petroleum as 2500, the amount present was calculated.

Vitamin A was estimated by two methods. (1) a 3-5 ml portion of the filtrate after removal of sterols was taken to dryness *in vacuo* at room temperature in a hard glass test tube. 0.5 ml chloroform was added to the residue, 4.0 ml SbCl_3 reagent run in and the mixture placed immediately in a 2 cm cell on the Hilger Nutting spectrophotometer and the extinction of the blue solution (maximum observed at 617 $\text{m}\mu$) measured, from which the concentration in μ could be calculated using a conversion factor of 550. (2) The ultraviolet absorption spectrum was recorded and using the three point method of correction for irrelevant absorption (Morton & Stubbs, 1940) the true absorption due to vitamin A was calculated, from which the concentration in μ was calculated, using a conversion factor of 1800.

RESULTS

Preliminary qualitative experiments with the SbCl_3 reagent on the non saponifiable matter from intestinal extracts indicated that vitamin A was present in the intestine during the absorption of carotene and that the maximum amount of vitamin A occurred 6-8 hr after dosing. No detectable quantities of vitamin A were found in the contents of the lumen of the small intestine or stomach. The quantitative results for the young rats fed with a solution of crystalline β carotene and for the control animals given the carotene rich diet are shown in Table 1.

The small intestines of the vitamin A depleted animals and of the controls (Exps II and III), which had a high liver reserve of vitamin A formed entirely from a carotenoid source, were examined for vitamin A. Both the SbCl_3 test and the correction procedure failed to demonstrate its presence, even in traces. In the remaining experiments (IV-XI), where β carotene was in process of absorption, vitamin A, characterized by its ultraviolet absorption spectrum and by the colour test with SbCl_3 , was always found in the gut wall. A typical absorption curve and the corrected vitamin A curve are shown in Fig 1. The extracts from all seven groups of animals gave similar curves capable of correction by the three point fixation method, and also the blue colour (absorption max., 617 $\text{m}\mu$) characteristic of vitamin A with the SbCl_3 reagent.

The vitamin was determined by both methods of analysis and in general, close agreement (columns 7 and 8, Table 1) was obtained for the amount present in spite of the large corrections which had to be applied to the ultraviolet absorption curves in the second method. The amount of vitamin A found was very small. In an attempt to increase the yield of vitamin A, the small intestines of the remaining rats were allowed to take up β carotene

Table 1 Vitamin A formed in the intestine of the rat during absorption of carotene

Exp	No of rats	β Caro tene given (mg /rat)	Time after dosing (hr)	Incu bation time (hr)	Intestine						Liver	
					Lumen		Gut wall				Caro tene (μ g)	Vitamin A (1 u /rat)
					Caro tene	Vitamin A	Caro tene (μ g)	Vitamin A (1 u)				
								U V	SbCl ₅			
I	4	0	—	—	—	—	0	0	—	0		
II	5	(1)*	—	—	—	0	(11)*	0	0	(111)*	6644	
III	5	(1)	—	—	—	0	(11)	0	0	(111)	2465	
IV	5	2.5	20	—	+ve	Nil	3	—	2	5	360	
V	3	2.5	16	—	+ve	Nil	2	—	3	5	70	
VI	4	5.0	7	—	+ve	Nil	5	10	20	-ve	49	
VII	4	5.0	7	—	+ve	Nil	34	35	28	2	108	
VIII (a)	4	5.0	6	—	+ve	—	30	6	6	1	25	
VIII (b)	5	5.0	7.5	3	+ve	—	35	10	12	11	80	
IX	1	10.0	6	15	+ve	—	20	10	—	—	—	
X	1	14.0	7.8	20	+ve	—	—	22	21	18	360	
XI	1	14.0	8.0	20	+ve	—	—	20	18	16	270	

* (i) Diet containing mixed carotenoids, (ii) trace of yellow pigment not carotene, (iii) trace of yellow pigment

under physiological conditions. They were then removed intact from the animal at the peak period of absorption (6–8 hr after dosing) and incubated in Ringer solution. In Exp IX the vitamin A

DISCUSSION

The preparation of an extract from the small intestine which gives both a correctable ultraviolet absorption curve for vitamin A and a strong colour test at 617 m μ , compared with control extracts which gave neither, is taken as unequivocal proof of the presence of vitamin A in the intestinal wall of the rat during the absorption of β carotene. Furthermore, in Exps VI–VIII c 10–30 i u of vitamin A were found in the intestine of each animal killed 6–8 hr after dosing with β -carotene, and the liver reserves of vitamin A were low (25–108 i u /liver), whereas in Exps IV and V on rats killed 20 and 16 hr respectively after dosing, the amount of vitamin A in the intestines was just detectable, i.e. 2 and 3 i u, and the liver reserves were 360 and 70 i u respectively. These results considered together with those of Exps II and III* can only be satisfactorily explained on the basis that vitamin A is formed from the β carotene in the intestine and does not originate in the liver. The result obtained in Exp VIII where vitamin A in the intestines was greater after incubation compared with control tissue from similarly dosed animals supports this view.

The absence of detectable quantities of vitamin A from the contents of the lumen and stomach during

* Although in the final analysis no vitamin A was detected in these tissue extracts, it is possible that the original extracts contained traces (≤ 5 i u), but the process of saponification and particularly that of chromatography of the vitamin A under the best conditions always involves a slight loss of vitamin. The loss, however, ought to be of the same order for each tissue examined as the technique was always the same.

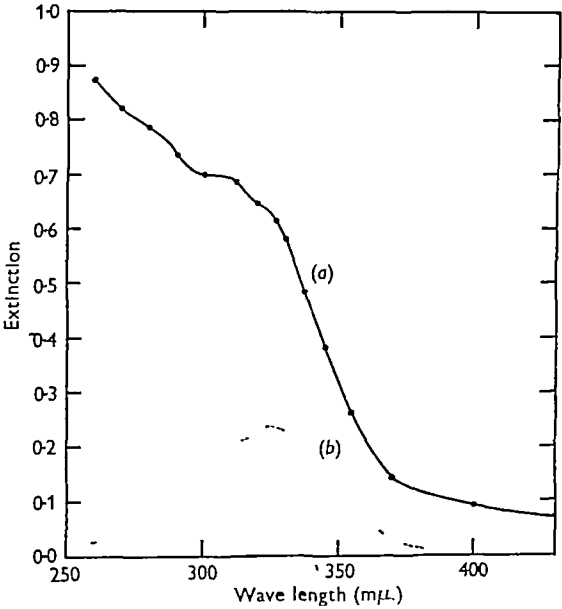


Fig 1 (a) Ultraviolet absorption spectrum of the gut wall extract (in methanol) of the carotene fed rat, and (b) the corrected vitamin A curve (E_{corr} 325 m μ . = 0.238 equivalent to a total of 175 i u)

content of the incubated gut walls is greater than that of the non incubated, but the increase is not sufficiently marked to assert definitely that *in vitro* conversion had taken place.

the absorption of the β carotene implies that the latter is converted into vitamin A on entering the gut wall. However, Thompson, Ganguly & Kon (1947) have reported vitamin A in the contents of the small intestine of the carotene fed rat in amounts comparable to that observed in the gut wall. In contrast to our result above, this could indicate that the carotene is broken down to vitamin A before it reaches the gut wall. It is, perhaps, possible that the vitamin found in the lumen contents arises from mucosal cells removed in the washings by friction when the intestines were being flushed out with ether. On the other hand, the observation (Glover *et al.* 1948) that vitamin A aldehyde, a possible intermediate in the breakdown of β carotene, is reduced to vitamin A immediately on entering the gut wall, supports the suggestion that this organ is responsible for the breakdown of β carotene. Furthermore, the coexistence of β carotene and vitamin A in the gut wall, coupled with the fact that much less β carotene is detected in the liver than in the wall of the small intestine during the absorption of the provitamin, strongly indicates that the latter organ is responsible for the conversion of β carotene to vitamin A.

The above work, with that of Mattson, Mehl & Deuel (1947) and Wiese, Mehl & Deuel (1947) on the rat, Thompson *et al.* (1947) on the rat and pig, and Goodwin & Gregory (1948) on the goat, establishes the important fact that the main site of conversion of the provitamin A, carotene, to vitamin A is the small intestine of the animal. The results bear out in full the idea that the failure of earlier workers to detect vitamin A in the intestine after feeding β -carotene was due to the relatively slow rate of conversion and rapid removal of the vitamin A formed from the intestine.

One main problem which has so far defied satisfactory explanation is the variation in the relative amounts of carotene and vitamin A in the blood and liver of different species. The differences can, however, be considered as dependent on the rate of intake of carotene and the efficiency or abundance of an enzyme system effecting the conversion in the intestinal wall. If the carotene intake is large, the

system will not be able to break down all the carotene and some will pass into the bloodstream and hence to the liver. Once it has passed the intestinal wall further conversion of carotene to vitamin A is improbable. It would seem that the widely varying ratios of carotene to vitamin A in the blood serum or liver of different animals are due to several factors: (a) varying levels of intake and variable success in presenting food carotene for assimilation, (b) variable efficiency* for its conversion into vitamin A in the intestinal wall, and (c) differences in the rates of withdrawal† of the provitamin and the vitamin from the blood.

SUMMARY

1 The small intestine of the rat fed on a carotene rich diet for 3 months and then placed on a vitamin A free diet for 4 days prior to examination does not contain a detectable amount of vitamin A, even though there is a moderate reserve of the vitamin in the liver.

2 When the absorption of a single dose of carotene by vitamin A depleted animal is maximal (approx. 6 hr after dosing), vitamin A, demonstrated by two independent methods, is present in the gut wall in an amount comparable to that in the liver, and the gut wall contains an appreciable quantity of carotene, although there is only a small or negligible amount in the liver.

3 These facts establish the wall of the small intestine as the locus of the transformation of carotene into vitamin A.

4 An explanation is suggested for the differences observed in the relative amounts of vitamin A and carotene in the blood and livers of different animal species.

We are indebted to the Medical Research Council and the Ministry of Food for grants in aid of these investigations.

* As the reaction may be an oxidation process, the efficiency will depend not only on the abundance of the enzyme present, but also on the hormonal activation of the enzymes responsible for the oxidation.

† Withdrawal includes physiological utilization and oxidative wastage.

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The Use of Radioactive Isotopes in Immunological Investigations

2 THE FATE OF INJECTED ^{32}P CONTAINING PROTEINS

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Isotopic tracers can be of considerable value in immunochemical investigations. ^{15}N has been used, for example, in studies on the formation and circulation of antibodies (Schoenheimer, Heidelberger, Rittenberg & Ratner, 1941; Heidelberger, Treffers, Schoenheimer, Ratner & Rittenberg, 1942). Radioactive isotopes of P and S have been used to determine the amount of tracer containing antigen in antigen antibody precipitates (Boursnell, Dewey, Francis & Wormall, 1947), and in investigations on the lipid P and vitellin P in lipovitellin anti-lipovitellin precipitates (Francis & Wormall, 1948). Libby (1947) and Libby & Madison (1947) have recently used ^{32}P containing tobacco mosaic virus to study the rate of breakdown of this antigen in the animal body. There are obviously many other ways in which labelled antigens and antibodies can be used in the field of immunology.

In the present investigation, a preliminary study has been made of the fate of injected ^{32}P containing antigens, phosphorylated human serum proteins and vitellin, to determine the length of time these antigens remain in the blood stream after injection.

METHODS

Phosphorylated proteins

Preparation of phosphoryl chloride containing ^{32}P Radioactive sodium phosphate was diluted with ordinary phosphate and precipitated as $\text{Ca}_3(\text{PO}_4)_2$ (0.9 g). This was dried and intimately ground with 0.9 g of HCl-extracted charcoal (Merck). The mixture was thoroughly dried at 150° and then heated in a dry all glass Pyrex apparatus in a metal bath at 300° , with dry air drawn through the $\text{Ca}_3(\text{PO}_4)_2$ charcoal mixture to remove traces of moisture. A weighed receiver was connected and cooled in a CCl_4 solid CO_2 mixture at about -25° and dry COCl_2 was passed through the mixture at $300-360^\circ$ until the reaction ceased. The residual $\text{Ca}_3(\text{PO}_4)_2$ charcoal mixture was removed and finely ground to break up the CaCl_2 layer which had formed over the remaining $\text{Ca}_3(\text{PO}_4)_2$, it was then reheated in a current of dry air and COCl_2 passed through as before. When this reaction had ceased, the POCl_3 in the receiver was warmed to remove COCl_2 and distilled (Yield 0.44 g, 49% of theory).

Preparation of phosphorylated human serum proteins Human serum (6.5 ml.) was phosphorylated with 0.229 g of $^{32}\text{POCl}_3$ (i.e. a POCl_3 /protein ratio of 1:2) as described previously (Boursnell, Dewey & Wormall, 1948). In this

experiment the proteins were not separated by precipitation with acid, but the solution was dialyzed at about 4° against 0.9% (w/v) NaCl until free from dialyzable phosphate, this product, which had a P/N ratio of 0.085, was treated with 0.05 vol of 5% (w/v) phenol and used for injection, a similar preparation used for the precipitin tests had a P/N ratio of 0.065

Treatment of the blood samples

Three rabbits each received an intravenous injection of ³²P containing phosphorylated human serum proteins, and blood samples (a little over 5.5 ml at each bleeding) were taken at intervals from the ear not used for the injection. A radioactivity determination was made on 0.50 ml of each blood sample, and 5.0 ml were centrifuged and the separated cells and plasma treated as follows

Cell fraction The cells were washed three times with 3 ml. of 0.9% NaCl each time, and a radioactivity determination was made on the whole sample

Plasma A radioactivity determination was made on 0.50 ml. of the plasma, and the remainder was used for separation of the proteins as follows -

(a) *Ethanol ether precipitation* Plasma (1.0 ml) was added to 6 ml. of cold ethanol ether (7:3 v/v) mixture, kept at 0° for 2 hr and then centrifuged. The precipitate was washed twice with 6 ml. of cold ethanol ether mixture each time, and three times with 6 ml. of cold ether. It was then Soxhlet-extracted with two 100 ml portions of CaCl₂ dried ether for 2 hr each time, and radioactivity determinations were made on the dried product. Determinations made on a few of the ethanol ether extracts showed that these contained no significant amount of ³²P

(b) *Trichloroacetic acid precipitation* Plasma (1.0 ml) was added to 4.0 ml of cold 10% (w/v) trichloroacetic acid. After 2 hr at 4° the precipitated proteins were centrifuged and washed three times with 3 ml. of 8% (w/v) trichloroacetic acid each time. Radioactivity determinations were made on the dried proteins

Determination of radioactivity

The samples were incinerated with HNO₃, Cu and NaClO₄, as described previously (Boursnell, Francis & Wormald, 1946a). The resulting pyrophosphate was hydrolyzed by boiling for 10 min with approx N HCl, and KH₂PO₄ equivalent to 100 mg of Mg(NH₄)PO₄ was then added. The phosphate was separated as ammonium phosphomolybdate, and converted to Mg(NH₄)PO₄, this was dried at 105-110° and a weighed amount transferred to a ring (1.7 cm diameter) mounted on a brass plate. The precipitate was spread evenly and protected by a few drops of dilute collodion solution in ethanol ether. Radioactivity determinations were carried out as described for ³²S by Boursnell *et al* (1946a), using the counter system described by Banks (1946)

Lipovitellin and vitellin

Preparation of antigens

Lipovitellin A solution of radio lipovitellin in 10% (w/v) NaCl was prepared from eggs laid by hens injected with ³²P containing neutralized sodium phosphate, as described previously (Francis & Wormald, 1948)

Vitellin A solution of radio lipovitellin (c 6% in 10% NaCl) was poured into 5 vol of ethanol ether (7:3) mixture at -15°. The mixture was centrifuged at once and the precipitate was washed once with ethanol ether and twice with ether, all operations being carried out at between -15 and -5° and as rapidly as possible. Most of the adhering ether was removed by rapid evacuation and the protein was washed once with ice cold water, it was then dissolved in a minimal amount of dilute NaOH and precipitated with dilute acetic acid, or dissolved in saturated urea solution and dialyzed. The products were emulsified with 0.9% NaCl solution with the addition of a few drops of 0.1 N NaOH and strained through muslin to give a fine cream suitable for injection

Preparation of materials for radioactivity determinations

Blood Blood from the marginal vein of the ear not used for the injection was collected in tubes containing oxalate at intervals after the injection of lipovitellin. The rabbits injected with vitellin died about 1 min after the injection and in these cases blood was then taken from the heart as quickly as possible. Whole blood samples (3 or 5 ml), or the separated plasma and cells, were evaporated to dryness several times with conc HNO₃ (2-3 ml) in 50 ml flasks until a clear straw coloured liquid was obtained. A further evaporation with HNO₃ plus 1-2 ml of 10% (w/v) NaClO₄ completed the destruction of all organic matter

Tissues The whole organs (liver, spleen, heart, lungs and kidneys) were removed immediately after the death of the rabbits injected with vitellin, these organs were dried with filter paper, weighed, dried at 100-150° and ashed by heating in a muffle furnace for about 6 hr at 400° and a further 6 hr at 700-800°

Radioactivity determinations Preliminary experiments showed that where the residue from the incineration of a sample was less than 100 mg, accurate radioactivity determinations could be made without separating the phosphate, these residues were dissolved in water containing a few drops of 2N HCl and the solution evaporated directly on copper or nickel holders. With larger residues, the material was dissolved in a mixture of N HCl and N HNO₃ (occasional insoluble fractions having been found to have no detectable radioactivity), and the solution boiled to hydrolyze pyrophosphoric acid. The phosphate was then precipitated with magnesia mixture, and a sample (up to 100 mg) spread on a nickel holder for radioactivity determination. All the results recorded in the tables are average values for two or more determinations

RESULTS

Injection of phosphorylated proteins

The serum proteins are strongly antigenic and, since phosphorylation can readily be effected at low temperature and at about pH 8, it was thought that phosphorylated human serum proteins might be suitable for immunological investigations. They are relatively non toxic and can be injected in amounts sufficient to permit one to trace the antigen after its injection. The ³²P available for some of our experiments was rather small, but the radioactivity of the

phosphorylated proteins used here was high enough to allow reliable determinations to be made on small blood samples taken from the rabbits at intervals. In this experiment the mixed phosphorylated proteins were freed from inorganic phosphate by dialysis, but were not otherwise purified or separated, in order to minimize as far as possible the chemical changes effected in the protein antigen.

The results of an experiment with three rabbits showed (Table 1) that the injected antigen did not remain long in the blood stream. Assuming complete mixing of the injected material with the circulating blood, and assuming a blood volume of 50 ml/kg of body wt (the approximate average for the blood volume calculated from values quoted by Erlanger (1921) for the rabbit), the radio P content of the blood of the injected animals would be about 8.6 $\mu\text{g/ml}$ shortly after the injection. The average values observed were 6.4 μg after about 10 min, 2.0 μg after 3 hr, and 0.5 μg of radio P/ml of whole blood 24 hr after the injection. As expected, the greater part of the ^{32}P was present in the plasma in the early stages of the experiment, the cell fraction of the blood contained only a very small amount 10 min and 3 hr after the injection. Determinations were not made on the cell fractions of later blood samples, since it was thought that any ^{32}P present in these cells might have originated from inorganic phosphate removed from the phosphorylated proteins. It will be observed that the sum of the radio P contents of the plasma (adjusted on the basis of a plasma volume of 55–60% of the whole blood volume) and the blood cell fraction does not quite agree in most cases with the value for whole blood. This might have been due to the loss of ^{32}P containing compounds during the washing of the cells.

In some cases ^{32}P determinations were made on the proteins separated from the plasma samples either by treatment with ethanol ether or by precipitation with trichloroacetic acid, with the object of finding out how much of the blood or plasma ^{32}P was present in the proteins. The values obtained with these separated proteins were similar to the

corresponding plasma values recorded in Table 1, and these plasma radio-P values can therefore be regarded as a measure of the amount of phosphorylated human serum protein present. In other investigations it has been found (Boursnell *et al* 1948) that treatment with ethanol ether under the conditions of our experiments leads to precipitation of inorganic phosphate with the protein, but this does not appear to be a complicating factor here, the amount of radioactive inorganic phosphate liberated in the first few minutes and possibly even during the first 3 hr after the injection would probably be small, and in any case much of it would readily diffuse from the blood to the tissues.

In an attempt to obtain, by a method independent of radioactivity determinations, an approximate measure of the length of time the injected protein, or its dephosphorylated counterpart, remained in the blood, some of the plasma samples were tested with antisera to human serum proteins. Since our antisera to phosphorylated proteins were not specific for the phosphate containing groups of phosphorylated proteins, it was decided that serological tests should be made for the human serum protein part of the molecule. The results showed that significant amounts of human serum proteins, or their phosphorylated derivatives (since these are precipitated by antisera to human serum proteins) were present in circulation up to at least 5 days after the injection.

Injection of lipovitellin and vitellin

In view of the fact that the phosphate groups of phosphorylated proteins are fairly readily removed at 37°, injection experiments have been carried out with the more stable labelled antigens, lipovitellin and vitellin. Both compounds can be synthesized biologically and obtained after a moderate amount of chemical treatment and manipulation.

The results of experiments with lipovitellin showed (Table 2) that the injected compound rapidly left the blood, about 12% only remained in the blood after 5 min, 2–4% after 5 hr and about 2% 24 hr after the injection.

Table 1 *Radio-P content of whole blood, cells and plasma of rabbits after the injection of phosphorylated human serum*

(Three rabbits A, B and C, weighing 3.25, 2.95 and 2.95 kg respectively, each received an intravenous injection of 1.25 ml/kg of a solution of dialyzed phosphorylated human serum containing 346 μg radio P/ml and 0.13 microcuries of ^{32}P /ml.)

Time after injection (hr)	Radio P content ($\mu\text{g/ml}$ of whole blood or plasma, or in the cells from 1 ml of whole blood)								
	Whole blood			Cells (washed)			Plasma		
	A	B	C	A	B	C	A	B	C
0.17	5.3	6.8	7.0	0.07	0.03	0.03	7.7	10.2	—
3	1.7	2.2	2.1	0.06	0.08	0.13	2.3	2.4	3.1
24	0.4	0.4	0.6	—	—	—	0.3	0.4	0.5
96	0.2	0.2	0.2	—	—	—	0.0	0.0	0.1
144	0.1	0.2	0.1	—	—	—	0.0	0.0	0.0

Table 2 *Radio P content of blood of rabbits following the intravenous injection of ³²P containing lipovitellin*

(Rabbits nos 541, 544 and 529, of wt 2.4, 2.4 and 2.3 kg respectively, each received an injection of 3.0 ml of lipovitellin solution containing 4572 μ g of radio P. Rabbit no 541 received a further similar injection 11 days later.)

Rabbit no	Time after injection (hr)	Radio P content (μ g/ml of blood)			Total* radio P in the blood of the rabbit, as % of that injected
		Whole blood	Plasma	Cells	
541	0.08	4.2	3.6	1.3	11
	5	1.3	0.6	0.7	3.5
	24	0.7	<0.15	<0.6	2
	48	1.4	<0.2	0.7	3.5
(2nd injection)	0.25	2.7	—	—	7
	4	<1.2	—	—	<3
	24	0.9	—	—	2.5
544	0.08	4.9	3.5	—	13
529	c 0.08	5.0	3.4	—	13

* Assuming a blood volume of 50 ml/kg of body wt

Vitellin, the corresponding lipid-free protein, was more difficult to deal with since it is practically insoluble in water or dilute salt solutions. However, with the aid of a little very dilute NaOH and in other cases with saturated urea solution, the vitellin was converted into a fine cream for injection. Unfortunately, this suspension proved very toxic to the two rabbits used for this experiment, and they both died in just under 1 min. after the injection. By withdrawing blood from the heart and removing selected organs at once, material was obtained for a study of the distribution of the injected antigen. The results (Table 3) showed that a considerable amount, possibly as much as half of the injected antigen, had left the blood stream in the first minute after the injection, most of the missing antigen being

found in the liver and lungs. Calculations have shown that the radio P in the blood present in the lung and liver samples analyzed could account for a small fraction only of the radio P content of these organs.

DISCUSSION

Table 3 *Radio P content of the blood and some organs of rabbits shortly after the intravenous injection of ³²P-containing vitellin*

(Rabbits nos 634 and 635, each of 2.7 kg, received 5.0 and 4.0 ml respectively of a solution or suspension of radio vitellin, the amount of radio-P injected was 2575 and 2080 μ g respectively. Both rabbits died about 1 min. after the injection. Blood was then immediately taken from the heart, and the organs were excised as quickly as possible and treated as described in the text.)

	Radio P content			
	Rabbit no 634		Rabbit no 635	
	(μ g)	As % of radio P injected	(μ g)	As % of radio P injected
Blood	2025*	79*	1080*	52*
Kidneys	37	1.4	<10	<0.5
Lungs	481	19	339	16
Spleen	0	0	0	0
Heart	10	0.4	0	0
Liver	402	16	354	17

* Assuming a blood volume of 50 ml/kg of body wt, and uniform distribution of the radio P throughout the blood

The site of antibody formation in the animal body is not clearly established, but it is believed by many investigators that antibodies, which, according to the most widely accepted view, are essentially modified plasma globulins, are synthesized in the same place as are the normal plasma proteins. Madden & Whipple (1940), from the evidence of their own and other investigations, concluded that the liver takes an important part in the manufacture of the normal plasma proteins, and it is not unreasonable to believe that this organ is partly or wholly responsible for the synthesis of antibodies against foreign proteins and other antigens. There is also evidence that the reticulo endothelial system as a whole takes part in this synthesis (cf reviews by Landsteiner, 1945, pp 146-7, Boyd, 1943, pp 62-4).

Whatever the site of antibody formation, it would seem almost certain that the injected antigen, or some fairly large fraction of the molecule, will be located in these tissues at some time after its injection. Working on this hypothesis several investigators have examined various tissues at intervals after the injection of readily detectable antigens. Haurowitz & Breml (1932), for example, used an arsenic containing azoprotein antigen and found that most of the arsenic remained in the liver and 'marrow bones'. Other evidence of the localization of much of the injected antigen in the liver was obtained by Rous & Beard (1934) using ferromagnetic iron oxide and by Sabin (1939) with the aid of a coloured azoprotein.

In our experiments we have used ³²P containing protein antigens, although these are not ideal labelled

proteins for such an investigation. The phosphorylated proteins, like the azoproteins, are artificial complexes, and most of the linkages between the introduced phosphate groups and the protein molecule are fairly readily split at 37° (Heidelberger, Davis & Treffers, 1941, Bournsnel *et al* 1948), this, together with the fact that the liberated inorganic phosphate will undoubtedly take part in metabolic reactions unrelated to antibody formation, renders results obtained with phosphorylated proteins of limited value. The localization of ^{32}P in any tissue after the injection of these proteins should only be regarded as being immunologically significant if it occurs fairly soon after the injection, though some of the phosphate groups may remain attached to the protein for some considerable time after the injection. The use of vitellin and lipovitellin is not open to the same objections, since these are natural proteins containing firmly bound phosphate. On the other hand, they are insoluble or almost insoluble in water and dilute salt solutions, though this is not an insuperable difficulty in experiments involving the tracing of injected antigen. A much better antigen for these investigations is the $\beta\beta'$ -dichlorodiethylsulphone protein compound (Berenblum & Wormal, 1939, Bournsnel, Francis & Wormal, 1946*b*), and now that supplies of ^{35}S have again become available in this country our investigations with this antigen are being continued.

The results of the experiments described here have shown that the injected antigens are rapidly removed from the circulating blood, and the two experiments with injected vitellin (Table 3) show that much of the antigen is removed by the liver during the first minute or so after the injection. (The presence of a considerable amount of ^{32}P in the lungs in this experiment is not regarded as necessarily significant, for it seems probable that the vitellin particles injected, or those precipitated when the suspension was injected into the vein, were unable to pass through the lung capillaries.) This removal of injected foreign particles by the liver is similar to that observed by Jones, Wrobel & Lyons (1944) and Dobson, Kelly, Jones & Gofman (1947), in their experiments with ^{32}P containing chromic phosphate, this injected compound became localized specifically in the liver and spleen, and practically all was removed in a single passage of the blood through the liver. Similar results were obtained with other radioactive inorganic colloids (Dobson *et al* 1947), but in our experiments the injected protein particles were not taken up by the spleen during the first minute or so after the injection. It is possible that the spleen may take up injected antigens or other colloids at a later stage, but the results obtained by Sheppard, Wells, Hahn & Goodell (1947) suggest that there may be fundamental differences in the phagocytic removal of different injected colloids. Using radio-

active isotopes these authors found, for example, that the injection of colloidal gold into men and dogs resulted in a high concentration of this element in both the liver and spleen, whereas the injection of colloidal manganese dioxide led to a high concentration of manganese in the liver but not in the spleen.

Specific deposition of radioactive colloid in the liver, and the resulting specific irradiation of that organ, led to a marked depression of deoxyribose nucleic acid metabolism (Jones, Kelly & Lawrence, 1947). One of the objects of our own investigations with ^{32}P containing lipovitellin and vitellin was to obtain specific irradiation of the reticulo endothelial system and any other tissue in which the injected antigen is localized. The relatively short half life period of ^{32}P (14.3 days) and the probability that the phosphoproteins will be slowly hydrolyzed in the tissues may render these egg yolk proteins specially suitable for this type of investigation. For some of these experiments we propose to use ^{32}P containing bacterial nucleoproteins, for preliminary experiments made in collaboration with Prof L P Garrod have shown that appreciable amounts of ^{32}P can be introduced into protein fractions of streptococci and coliform organisms.

SUMMARY

1 ^{32}P containing proteins have been used to study the fate of intravenously injected antigens. The antigens used were lipovitellin and vitellin obtained from eggs laid by hens injected with ^{32}P -containing inorganic phosphate, and artificially phosphorylated human serum proteins.

2 The injected proteins quickly disappeared from the blood stream. Less than one quarter of the injected phosphorylated human serum proteins was detectable in the blood 3 hr after the injection, and the disappearance of vitellin and lipovitellin was even more rapid, since only about one eighth of the injected protein could be detected in the blood 5 min after the injection.

3 Appreciable amounts of ^{32}P were found in the liver and lungs of two rabbits 1 min after the injection of a suspension of vitellin. The presence of this material in the lungs was possibly due to the retention, by the lung capillaries, of vitellin which was precipitated when the material was introduced into the blood stream.

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***p*-Ethylphenylsulphuric Acid in Goat Urine**

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Although it was suggested by Baumann in 1879 that *p* ethylphenol might be formed in the animal body by the degradation of tyrosine, the presence of this substance was not reported until 1927, when Walbaum & Rosenthal (1927) and Pfau (1927), isolated it from the dried scent glands of the beaver. More recently, Lederer (1943, 1946) isolated *p*-ethylphenol from an extract of acid hydrolyzed pregnant mare urine.

In the present work the isolation of *p* ethylphenyl sulphuric acid from urine as the potassium salt is reported for the first time. This substance was initially obtained from the urine of an ovariectomized goat, which had received large doses of progesterone and hexoestrol. Subsequently it was also isolated from the urine of a normal goat.

In view of the belief (Williams, 1947) that *p* cresol is quantitatively the most important phenol in the urine of vertebrates, it is noteworthy that no clear cut evidence has been obtained for the presence of *p* cresyl sulphuric acid in the urine examined. The fact that the derivatives of *p* ethylphenol, prepared from the hydrolysis product of the sulphate, required frequent recrystallization before constant melting points could be obtained, might suggest that the isolated substance was contaminated with

appreciable amounts of the sulphate of *p* cresol or of other phenols. Nevertheless, the present work indicates that in the goat *p* ethylphenylsulphuric acid is excreted in larger amount than *p* cresylsulphuric acid.

EXPERIMENTAL

The experimental animals were mature ovariectomized goats on a diet of hay and a mixture of bran, linseed cake, oats and beans. For the purpose of lactation studies under the direction of Dr S J Folley at the National Institute for Research in Dairying, they received 1 mg hexoestrol and 40 mg progesterone daily. Dr Folley and Mr A T Cowie kindly arranged for the collection of urine, and for its dispatch to this laboratory.

The urine was collected quantitatively under butanol. Daily collections were stored in the refrigerator. The pooled 7 day collections received were worked up for conjugated substances. Normal goat urine was collected similarly from an animal receiving no treatment.

Extraction of urine

Urine (3.5 l) from a treated goat was acidified to pH 3 with HCl, and rapidly extracted with butanol at low temperatures. The butanol extract was washed with $\pi/3$ NaOH, and water, and evaporated to dryness under reduced pressure. The residue was dissolved in water, chilled to 0°,

acidified to pH 2 with HCl, and rapidly extracted with chilled ether. The ether washed aqueous phase was made alkaline to litmus with NaOH, and extracted with butanol. After washing with water the butanol was evaporated *in vacuo*, leaving 5 g of light brown solid.

Preparation of potassium and p toluidine salts

The above-described solid was dissolved in a minimum of water, and an equal volume of saturated potassium acetate solution added. The crystalline precipitate formed was washed twice with half saturated potassium acetate solution, and dissolved in 200 ml. butanol. After washing several times with water, the butanol was evaporated *in vacuo*, leaving 1.933 g of nearly white crystals. After crystallization from 4% (v/v) aqueous acetone, crystals melting at 264–266° (corr) were obtained.

The material thus obtained dissolved readily in water, and gave a test for sulphate after warming with HCl. Millon's reagent gave a positive reaction after heating several minutes at 100°. The Tollens' reaction for glucuronic acid was negative. N, P and halogens were absent.

After drying for several days *in vacuo* over P_2O_5 the following analytical results were obtained: C, 40.05, 40.09, H, 3.86, 3.79, S, 14.0, 13.9, K, 16.5, 16.8, *potassium ethylphenylsulphate* ($C_8H_9SO_4K$) requires C, 39.98, H, 3.78, S, 13.34, K, 16.27%.

0.154 g of the above described potassium salt, and 0.225 g of *p*-toluidine hydrochloride were dissolved separately in 5 ml lots of water. The two solutions were warmed to 50° and mixed. The precipitate of *p*-toluidine salt formed was recrystallized three times from water at 50°, giving a product melting at 164–166° (corr) (decomp). Mixed with a sample of authentic *p*-toluidine salt of *p*-ethylphenyl sulphuric acid, there was no depression in melting point. Found: C, 58.25, 58.24, H, 6.02, 6.02. *p*-Toluidine salt of *p*-ethylphenylsulphuric acid ($C_{15}H_{19}NO_4S$) requires C, 58.24, H, 6.19. Heating above 50° caused marked decomposition of the *p*-toluidine salt of the sulphate.

Hydrolysis of the potassium salt

Hydrolysis was effected by boiling the potassium salt with approximately N HCl for 20 min., cooling, and extracting with ether. The residue obtained on evaporation of the washed ether was distilled at 100° and 0.1 mm to give a white crystalline solid. This had a strong phenolic smell and gave a blue colour, turning to dirty green, with ferric chloride solution.

Benzoylation by the Schotten-Baumann method yielded a product which, after six recrystallizations from moist ethanol, melted at 59–60° (corr). Mixed with authentic *p*-ethylphenyl benzoate (m.p. 60°, corr) the m.p. was 58–60° (corr). Mixed with authentic *p*-cresyl benzoate (m.p. 71°, corr) the m.p. was 53°. Found: C, 79.64, 79.49, H, 6.06, 6.05. Calc. for *p*-ethylphenyl benzoate ($C_{15}H_{15}O_2$): C, 79.64, H, 6.23, for *p*-cresyl benzoate ($C_{14}H_{13}O_2$): C, 79.23, H, 5.70%.

The oxyacetic acid derivative of the ether soluble hydrolysis product, after five recrystallizations, melted at 94–95° (corr). Mixed with authentic *p*-ethylphenoxycetic acid (m.p. 95–96°, corr) the m.p. was 94–95° (corr) and mixed with authentic *p*-methylphenoxycetic acid (m.p. 136°, corr) the m.p. was 97–98° (corr). 5106 mg dissolved in water required 1.17 ml 0.0236N NaOH giving an equivalent of 184.9. Calc. for *p*-ethylphenoxycetic acid 180.2.

Normal goat urine

By the processes described above 5.41 normal goat urine gave 1.15 g potassium salt. The *p*-toluidine salt prepared from this gave C, 58.46, 58.12, H, 6.11, 5.96. *p*-Toluidine salt of *p*-ethylphenyl sulphuric acid ($C_{15}H_{19}NO_4S$) requires C, 58.24, H, 6.19%. The benzoate obtained from the ether soluble hydrolysis product, after six recrystallizations from ethanol, melted at 57° (corr). Mixed with authentic *p*-ethylphenyl benzoate (m.p. 60°, corr) the m.p. was 56–57° (corr). After seven recrystallizations from water the oxyacetic acid melted at 95°, mixed with authentic *p*-ethylphenoxycetic acid the m.p. was 94–95°.

SUMMARY

1 The isolation of *p*-ethylphenylsulphuric acid from animal sources, as the potassium salt, is reported for the first time.

2 The *p*-ethylphenylsulphuric acid was obtained from goat urine, in which it appears to be quantitatively the most important phenylsulphuric acid.

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Inhibition of Carbonic Anhydrase by Sulphonamides

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Mann & Keilin (1940) discovered that sulphanilamide and certain related substances are specific inhibitors of carbonic anhydrase. In subsequent work on this enzyme (reviewed by Davenport, 1946) these inhibitors have proved useful tools in the study of the physiological action of carbonic anhydrase. According to Mann & Keilin the NH_2 group of sulphanilamide is not concerned with the inhibitory action, for benzenesulphonamide and N^4 acetyl sulphanilamide are also powerful inhibitors. In contrast the SO_2NH_2 group is concerned, for no inhibitions were found with sulphanilamide derivatives of the type $R\text{SO}_2\text{NH}R'$ to which most of the sulphonamide drugs belong.

Relatively little is known on the quantitative comparison of the inhibitory powers of different sulphonamides. Davenport (1945) has shown that in dilute solutions of carbonic anhydrase the expression

$$\frac{\text{uninhibited fraction of carbonic anhydrase}}{\text{inhibited fraction of carbonic anhydrase}} \times \text{concentration of inhibitor}$$

is constant, which is to be expected if the inhibition is due to a simple reversible reaction between carbonic anhydrase and the inhibitor. Davenport determined the value of this expression (K , representing the inhibitor concentration when the inhibition is 50%) for sulphanilamide and for thiophene 2 sulphonamide in dilute enzyme solutions, by measuring the inhibition at different concentrations of the inhibitor. For sulphanilamide, K was $1.0 \times 10^{-6} \text{ M}$ at 0° and $1.8 \times 10^{-6} \text{ M}$ at 10° , for thiophene 2 sulphonamide it was $1.6 \times 10^{-7} \text{ M}$ at 0° and about the same at 10° .

This paper is an account of a survey of the inhibitory power of twenty-five sulphonamides tested in connexion with the experiments on carbonic anhydrase described by Krebs & Roughton (1948). The concentrations causing an inhibition of 50% in various conditions were ascertained.

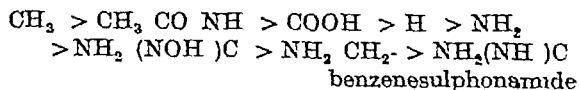
EXPERIMENTAL

The inhibition of the reaction between bicarbonate and phosphate buffer was measured with the technique described by Krebs & Roughton (1948). The carbonic anhydrase preparation was a chloroform ethanol extract of ox blood containing 9.2 mg dry matter/ml. For the test

it was diluted 80 or 160 times and 0.1 ml of the diluted solution, containing 0.012 or 0.006 mg dry matter, was added to the main compartment. The inhibitor, in a volume of 0.1 ml, was added to the main compartment. Preliminary experiments established the order of magnitude required to produce a 50% inhibition and several measurements were then made at the critical inhibitor concentration. Davenport's formula was used in calculating the concentration causing an inhibition of 50%. As pointed out by Roughton & Booth (1946) and Krebs & Roughton (1948) results often show considerable variations when the concentrations of carbonic anhydrase are very low, as was the case in the present experiments. All data given are based on at least three independent measurements but no great accuracy can be claimed. In general, the data obtained at 0° are more reliable than those obtained at 15° . The error at 0° is estimated at $\pm 30\%$.

















RESULTS

Data obtained with the foregoing procedure are given in Table 1. At 15° the inhibiting power was, in all cases, lower than at 0° , the differences between the values at the two temperatures varying from substance to substance. The data for sulphanilamide agree well with Davenport's (1945) figures, whilst in the case of thiophene 2 sulphonamide the present figures are higher than those recorded by Davenport. The most powerful inhibitors were prontosil red and prontosil soluble which caused 50% inhibition at a molar concentration of about 10^{-8} . At 0° *p* toluidenesulphonamide, acetylsulphanilamide, *p* sulphonamidobenzoic acid, naphthalene-1 sulphonamide, naphthalene 2 sulphonamide and 2 chloropyridine 5 sulphonamide caused a 50% inhibition at molar concentrations between 1×10^{-7} and 2×10^{-7} , they are thus more powerful inhibitors than thiophene 2 sulphonamide. The comparison of several *p* substituted benzenesulphonamides shows that in this series the inhibiting power decreased in the following order



p Toluenesulphonamide was more active as an inhibitor than *o* toluenesulphonamide, N^4 acetyl sulphanilamide was more active than sulphanilamide, and benzenesulphonamide much more active

Table 1 *Inhibition of carbonic anhydrase by sulphonamides*

Substance	Formula	Concentration causing 50% inhibition ($10^{-7}M$)	
		At 0°	At 15°
A <i>p</i> Substituted benzenesulphonamides			
Benzenesulphonamide	 SO ₂ NH ₂	4.6	—
<i>p</i> Toluenesulphonamide	CH ₃  SO ₂ NH ₂	1.0	2.4
<i>p</i> Aminobenzenesulphonamide (sulphanilamide)	NH ₂  SO ₂ NH ₂	9.0	19
<i>p</i> Acetamidobenzenesulphonamide (<i>N</i> ⁴ acetylsulphanilamide)	CH ₃ CO NH  SO ₂ NH ₂	1.2	2.0
<i>p</i> Aminomethylbenzenesulphonamide (marfanil)	NH ₂ CH ₂  SO ₂ NH ₂	25	—
<i>p</i> Sulphonamidobenzamidine	NH ₂ (NH)C  SO ₂ NH ₂	41	—
<i>p</i> Sulphonamidobenzamidoxime	NH ₂ (NOH)C  SO ₂ NH ₂	11	—
<i>p</i> -Sulphonamidobenzoic acid	COOH  SO ₂ NH ₂	2.0	2.7
B Other aromatic sulphonamides			
<i>o</i> Toluenesulphonamide	CH ₃  SO ₂ NH ₂	6.3	7.7
<i>o</i> Sulphonamidobenzoic acid	COOH  SO ₂ NH ₂	5.3	15
Toluene ω sulphonamide	 CH ₂ SO ₂ NH ₂	150	—
Naphthalene 1 sulphonamide	 SO ₂ NH ₂	1.6	2.0
Naphthalene 2 sulphonamide	 SO ₂ NH ₂	1.0	2.7
Thiophene 2-sulphonamide	 SO ₂ NH ₂	3.0	3.8
2 Chloropyridine 5 sulphonamide	Cl  SO ₂ NH ₂	1.2	—
4-Sulphonamido 2' 4' diaminoazobenzene (prontosil red)	—	0.06	—
4' Sulphonamidophenyl 2 azo 7 acetamido 1 hydroxynaphthalene 3 6 disulphonic acid (Na salt) (prontosil soluble)	—	0.10	—
C <i>N</i> ² Substituted sulphonamides			
Sulphamezathine	—	2,200	4,200
Sulphapyridine	—	510	1,400
Sulphathiazole	—	6,600	7,700
Sulphaguanidine	—	300	650
Sulphacetamide	—	6,500	19,000
Sulphanilyl sulphanilate	—	12,000	21,000
<i>p</i> Toluenesulphonylglycine	CH ₃  SO ₂ NH CH ₂ COOH	4,400	7,600
D Aliphatic sulphonamides			
β Cyanoethanesulphonamide	CN CH ₂ CH ₂ SO ₂ NH ₂	380	—

than toluene ω sulphonamide. The one aliphatic sulphonamide which was tested was a relatively weak inhibitor.

Sulphonamide drugs of the type $R\text{SO}_2\text{NH}R'$ (sulphapyridine, sulphathiazole, sulphamezathine and sulphaguanidine) also inhibited carbonic anhydrase when high concentrations were tested. The concentrations required to produce a 50% inhibition were between 100 and 10,000 times higher than in the case of analogous compounds without a substituent attached to the N^1 atom. But even though the inhibitory capacity of these substances is smaller than that of other sulphonamides they have marked effects in molar concentrations of 10^{-3} or 10^{-4} . For example, sulphapyridine causes 73% inhibition (at 15°) when the molar concentration is 2×10^{-4} (or 5.2 mg/100 ml). These are concentrations which are reached in the blood plasma in the standard procedures of treatment. Much higher concentrations may occur in the urine and, if the drug is given orally, in the stomach and in the intestinal tract.

Inhibition of carbonic anhydrase in the presence of tissue constituents

The inhibition of carbonic anhydrase by a given concentration of inhibitor depends on the affinity of the inhibitor for the enzyme and for other substances in the solution (see Davenport, 1941, van Goor, 1946). In the very dilute solutions of carbonic anhydrase to which the measurements recorded in Table 1 refer the second factor was of minor importance. Animal tissues and body fluids may be expected to contain substances capable of combining with sulphonamides and the inhibition of carbonic anhydrase in its natural medium may therefore be smaller than the values given in Table 1 suggest. The following experiments were undertaken to assess the order of magnitude of the effect of tissue constituents on the inhibition of carbonic anhydrase.

Sheep kidney cortex was homogenized in a Waring Blender with 1 vol of 0.1M phosphate buffer of pH 7.0. The homogenate was heated for 1 hr in boiling water to inactivate carbonic anhydrase (heating to 70° proved sufficient). The heated material was mixed with an equal volume of 0.02M phosphate buffer of pH 7.0 and again homogenized. The resulting liquid contained 5.2% dry matter and had a pH of 7.0. For carbonic anhydrase experiments 2 ml. of the homogenate were placed in the main compartment of a Warburg cup, together with 0.2 ml. additional solutions (carbonic anhydrase, inhibitor solution, or water). The side arm contained 0.2 ml. 0.25M NaHCO_3 . After mixing, a total pressure of about 200 mm. was observed and experiments with inhibitors indicated that the heated homogenate still contained a measurable amount of carbonic anhydrase. To test the effect of inhibitors 0.1 ml. of a diluted (1:160) chloroform ethanol extract of ox blood was added to the main compartment, with and without the inhibitor to be examined.

The data obtained are given in Table 2. It will be seen that the concentration required to produce a 50% inhibition is in all cases increased by the presence of tissue homogenate. The extreme cases were those of prontosil red, where the concentration was 83 times, and *p* sulphonamidobenzoic acid where it was only 1.2 times as high as that required in the absence of tissue homogenate (at 0°).

DISCUSSION

Inhibitory effect in warm blooded animals under physiological conditions. Whilst the inhibitory action of the sulphonamides decreased in all cases with rising temperature, the temperature coefficient varied considerably from substance to substance. The manometric technique is not suitable for the exact measurement of the inhibition at body temperature owing to the relatively high rate of the uncatalyzed reactions of carbonic acid at that temperature, but an approximate estimate of the inhibition can be obtained by extrapolation from the

Table 2 *Effect of tissue homogenates on the inhibition of carbonic anhydrase by sulphonamides*

For experimental conditions, see text. The effect of tissue constituents is indicated by the ratio

$$\frac{C_H}{C_0} = \frac{(\text{concentration of sulphonamide causing 50\% inhibition with homogenate})}{(\text{concentration of sulphonamide causing 50\% inhibition without homogenate})}$$

Substance	Experiments at 0°		Experiments at 15°	
	C_H (10^{-3}M)	Ratio C_H/C_0	C_H (10^{-3}M)	Ratio C_H/C_0
Sulphanilamide	39	4.3	—	—
N^4 Acetylsulphanilamide	5.4	4.5	11	5.5
<i>p</i> Toluenesulphonamide	7.0	7.0	12.7	5.3
<i>p</i> Sulphonamidobenzoic acid	2.3	1.2	3.7	1.4
Thiophene 2 sulphonamide	8.0	2.7	8.0	2.1
Naphthalene 1 sulphonamide	10	8.3	—	—
Prontosil red	5	83	18	—
Sulphaguanidine	2300	7.7	—	—

data given for 0 and 15°. The effect of tissue constituents on the inhibition under physiological conditions will likewise have to be assessed by extrapolation. The measurements listed in Table 2 were carried out in a medium containing about one quarter of the amount of tissue material present in the intact organ. Moreover, the protein was denatured by heat treatment.

Choice of inhibitor for physiological experiments. Of the substances tested *p* sulphonamidobenzoic acid may have advantages over other substances in inhibitor experiments. Its powerful action is not much affected by temperature and by the presence of tissue constituents. The neutral sodium salt is very soluble in water (about 20 % w/v) and can therefore be added in concentrated solution. It is worthy of note that *p* sulphonamidobenzoic acid has been found in urine after administration of marfanil (Hartles & Williams, 1946) and of *p* toluenesulphonamide (Flaschen trager, Bernhard, Löwenberg & Schläpfer, 1934). In some experiments it may be disadvantageous that

the inhibiting principle is contained in an anion, as in this substance, in such cases benzene, *p* toluene, naphthalene or thiophene 2-sulphonamide may be preferable.

SUMMARY

1 The inhibitory action of twenty five sulphonamides on carbonic anhydrase was examined under varying conditions. Data are given for the concentration causing an inhibition of 50 % at 0 and at 15°, in dilute enzyme solution, and also in the presence of tissue constituents.

2 Relations between chemical constitution and inhibitory power and the suitability of various substances for inhibitor experiments are discussed.

The author is indebted to Dr James Walker, National Institute for Medical Research, London, N W 3, for samples of marfanil, *p* sulphonamidobenzamide, *p* sulphonamidobenzamidoxime, 2 chloropyridine 5 sulphonamide and β cyanoethanesulphonamide.

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Studies in the Biochemistry of Micro-organisms

79 FUSCIN, A METABOLIC PRODUCT OF *OIDIODENDRON FUSCUM* ROBAK PART I. PREPARATION, PROPERTIES AND ANTIBACTERIAL ACTIVITY

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Smith (1946), in a note on the occurrence of species of the micro fungus *Oridiodendron* Robak in Britain, states that 'the genus *Oridiodendron* was erected by Robak (1932) on three species which were all found growing on ground wood pulp in Norwegian mills. The original three species are *O. nigrum*, *O. fuscum* and *O. rhodogenum*. Later, Robak (cited by Meln & Nannfeldt, 1934, p. 440) described a fourth species, *O. griseum*, and a further addition to the genus was made by von Szilvinyi (1941) with his *O. flavum*'.

The purpose of the present communication is to describe the preparation, properties and antibacterial activity of a hitherto undescribed mould metabolic

product, isolated from two different strains of *O. fuscum*, for which the name *fuscin* is proposed. Both the strains were identified by Mr G. Smith and were isolated originally from British sources, one—strain Ag 112—by the late J. H. V. Charles from guncotton at Ardeer, Scotland, in 1928, the other—strain Camb 111—by Dr Alan Burges in Cambridge, England, in 1945, from the surface humus in a pine plantation.

It has been found that, when either of these strains is grown on Czapek Dox 5 % glucose solution, the yellow-orange culture filtrate after 28–30 days' incubation at 24° shows a marked antibacterial activity against *Staphylococcus aureus*. Aeration of

the clear culture filtrate (pH 5.0) from strain Ag 112 leads to the deposition of an orange red microcrystalline precipitate of crude fuscine, $C_{15}H_{16}O_6$, and the disappearance from the culture filtrate of most of its antibacterial activity. It has been proved that in this case the freshly prepared culture filtrate contains a mixture of fuscine with considerably larger amounts of its reduction product, dihydrofuscine, $C_{15}H_{18}O_6$, which on aeration is oxidized by an enzyme system to fuscine. Strain Camb 111 behaved rather differently, since aeration of its culture filtrate (pH 6.2) led to no deposition of fuscine. However, on acidification and ether extraction of this culture filtrate, fuscine was readily isolated and purified.

The isolation of fuscine and its reduction product dihydrofuscine from cultures of the same strain of *Oidiiodendron fuscum* lends further support to the growing belief that metabolic products of this type play some part in the oxidation-reduction mechanism of the moulds which produce them. Thus, from culture filtrates of *Aspergillus fumigatus* Fresenius, Anslow & Raistrick (1938) isolated fumigatin, 3-hydroxy-4-methoxy-2,5-toluquinone and the corresponding quinol, 3-hydroxy-4-methoxytoluquinol. Posternak (1939) obtained the substituted ditoluquinone, phenicin, and its leuco derivative tetrahydrophenicin from *Penicillium rubrum* Grasberger. Stoll, Ashley, Raistrick & Richards (1939) found that certain species in the *Aspergillus glaucus* series produce, along with physcion, 4,5-dihydroxy-7-methoxy-2-methylanthraquinone, two reduction products of physcion, i.e. the anthranols 4,5-dihydroxy-7-methoxy-2-methyl-9-anthranol and 4,5-dihydroxy-7-methoxy-2-methyl-10-anthranol, and Ashley & Raistrick (1938) separated from the mycelium of *Helminthosporium leersii* Atkinson yellow luteoleusin, $C_{26}H_{36}O_7$, and its colourless reduction product alboleusin, $C_{26}H_{40}O_7$. All these quinonoid metabolic products and their corresponding leuco derivatives have been shown to be interconvertible.

Fuscine crystallizes in brilliant orange diamond-shaped plates, m.p. 230°. Its chemistry and its derivatives and breakdown products, a number of which have been prepared, will form the subject of a future communication. Hence it will suffice here to state that it has many properties suggestive of a quinonoid structure. Thus it readily sublimes unchanged in a high vacuum, it dissolves in alkalis with the formation of intensely purple coloured salts, it is easily reduced to colourless dihydrofuscine by cold aqueous sodium hyposulphite or catalytically by hydrogen in the presence of a palladium catalyst, conversely, dihydrofuscine is readily reoxidized to fuscine by aeration of its solution in pH 9.2 buffer.

Fuscine has marked antibacterial activity, particularly against all the Gram positive bacteria tested, the growth of which it completely inhibits at con-

centrations of 1/80,000 to 1/1,280,000. It is much less active against Gram negative organisms. *Vibrio cholerae* was inhibited at 1/80,000, six other species at 1/5000 to 1/10,000, but four species, including two of *Salmonella* and two of *Shigella*, were not completely inhibited even at concentrations of 1/5000. Two species of mycobacteria were inhibited at 1/10,000 and 1/20,000, while it was inactive against a third species.

Several antibiotics, notably penicillin, streptomycin and patulin, are inhibited in the presence of thiol compounds, but the nature of this inhibition is not yet clearly understood. When a slightly alkaline solution of fuscine is treated with thioglycolic acid the initial intensely purple colour of the solution quickly disappears. Subsequent acidification of the almost colourless solution leads to the deposition of a microcrystalline precipitate which, on crystallization from hot water, gives colourless needles, m.p. 200°, the analysis of which corresponds to a complex of 1 mol fuscine + 1 mol thioglycolic acid + 1 mol water. It is perhaps somewhat surprising that the conversion of fuscine into this complex causes little change in its antibacterial spectrum.

EXPERIMENTAL

Cultures

Fuscine was prepared from two different strains of *Oidiiodendron fuscum* Robak. They bear the London School of Hygiene and Tropical Medicine (L.S.H.T.M.) catalogue numbers Ag 112 and Camb 111. Strain Ag 112 was used exclusively for the bulk production of fuscine.

Strain Ag 112 was isolated by the late J. H. V. Charles in April 1928 from guncotton in the research laboratories of Nobel's Explosives Co. Ltd., Ardeer, Scotland.

Strain Camb 111 was isolated by Dr Alan Burges, School of Botany, Cambridge in October 1945 from the surface humus in a pine plantation at Wangford Warren, near Brandon.

Both strains were identified by my colleague, Mr George Smith, who reports that 'growth is very slow, grey to fuscous, powdery, with some dirty brown pigment in the medium (Smith, 1948)'.

Cultural conditions

A volume of 350 ml Czapek Dox solution (glucose, 50 g, $NaNO_3$, 20 g, KH_2PO_4 , 10 g, KCl , 0.5 g, $MgSO_4 \cdot 7H_2O$, 0.5 g, $FeSO_4 \cdot 7H_2O$, 0.01 g, distilled water, 1 l.) was measured into each of a number of 1 l. conical flasks, and sterilized. The medium was inoculated with a heavy spore suspension prepared from 3-4-week cultures of *O. fuscum* strain Ag 112 grown on Czapek Dox agar slopes. Batches of 100 such flasks were incubated in the dark at 24° and were harvested after 28-30 days, when the surface of the mould mycelium was grey in colour, the reverse almost black, and the culture fluid orange to reddish brown. Crystals of fuscine were sometimes observed in or on the mycelium of older cultures (5-6 weeks).

Isolation of fuscine from the culture fluid

The culture fluid was separated by filtration from the mycelium. The culture filtrate (33–34 l) still contained from 2.8 to 3.0% of glucose, had almost invariably a pH of 5.0 and completely inhibited the growth of *Staphylococcus aureus* at a dilution of 1:80 in 2% glucose broth after 24 hr incubation. The filtrate, initially clear and orange in colour, although it was quite stable in the absence of air, gradually changed on exposure to air either at room temperature or even after chilling, to a dark brown and in the course of a few hours an orange red precipitate of crude fuscine began to separate. It will be shown later that the culture filtrate, before exposure to air, contained a mixture of fuscine with a much larger amount of its reduction product, dihydro-fuscine. Since dihydro-fuscine is relatively unstable because of the readiness with which it is oxidized to fuscine, the stable quinonoid form, the following procedure was adopted for isolation. The culture filtrate was distributed equally between four 10 l bottles. A vigorous stream of air was passed through the bottles for 10 hr at room temperature, and after standing overnight the resulting precipitate was separated by filtration through a layer of kieselguhr, washed well with water and dried. The crude material consisted of lens shaped, microscopic, orange brown crystals. The filtrate, now very dark, contained very little fuscine and was discarded.

The crude fuscine was purified by extraction of the dried fuscine kieselguhr powder with boiling ethanol (1300–1400 ml/100 flask batch). On cooling the ethanol solution after filtration from the kieselguhr, almost pure fuscine separated in shining, orange red, diamond shaped plates which were filtered off, washed with ice cold ethanol and dried. Evaporation of the ethanolic mother liquors and washings to about 75 ml. yielded a second crop, usually about 1 g/batch, of crystalline fuscine, of almost equally good quality.

In all, 25 batches of 100 flasks each were prepared and yielded a total of 242 g of crystalline fuscine (first and second fractions combined) which corresponds to an average yield of 9.68 g/batch or 0.315 g/l of culture filtrate. The maximum yield in any one batch was 12.4 g and the minimum 7.86 g.

Isolation of fuscine from *Oidiodendron fuscum*, strain Camb 111 Ten 1 l flasks, each containing 350 ml of Czapek Dox solution, were inoculated with *O. fuscum*, strain Camb 111, and incubated at 24°. When the flasks were harvested after 21 days they appeared very similar to those of strain Ag 112. The mycelium was grey with a dark reverse. The yellowish brown culture filtrate had the following characteristics: residual glucose, 3.2%, pH, 6.2, completely inhibited the growth of *Staph. aureus* at a dilution of 1:320.

The culture filtrate was vigorously aerated, but, unlike that from strain Ag 112, no precipitate was formed. The filtrate was therefore made acid to Congo red with HCl and extracted with two portions of ether each of 750 ml. On removal of the solvent from the orange brown extract there remained 0.55 g of a brown powder which was crystallized from hot ethanol. The resulting orange crystals had all the properties of fuscine from strain Ag 112, e.g. m.p. and mixed m.p. 230°, identical colour reactions with ethanolic FeCl₃ and with aqueous alkalis, and similar antibacterial activity against *Staph. aureus*.

Isolation of dihydro-fuscine from culture filtrates of *Oidiodendron fuscum*, strain Ag 112 The contents (about 2.3 l.) of seven 1 l flask cultures of *O. fuscum*, strain Ag 112, 28 days old, were filtered quickly on a Buchner funnel directly into conc. HCl (100 ml), and all subsequent operations were carried out, as far as possible, in an atmosphere of nitrogen. The acid filtrate was extracted three times with 500 ml. portions of ether. The combined ether extracts were concentrated to 10 ml and chilled overnight. The resulting crystals were collected and recrystallized from ether yielding 0.13 g of buff coloured prisms, m.p. 204°, not depressed on admixture with dihydro-fuscine, m.p. 206°, prepared by the reduction of fuscine (see p. 531). The combined mother liquors yielded on evaporation a further crop of crystals consisting of a mixture of colourless dihydro-fuscine and orange fuscine.

The oxidation of dihydro-fuscine to fuscine by aeration of culture filtrates of *O. fuscum*, strain Ag 112, appears to be effected by an enzyme system. Such a filtrate, 100 ml of which gave a good precipitate of fuscine on direct aeration, afforded no fuscine when treated as follows: (a) 100 ml were brought to the boil in an atmosphere of N₂, cooled and aerated for 16 hr, (b) 200 ml. were acidified with conc. HCl (10 ml), kept overnight and then carefully readjusted to pH 5.0 with conc. aqueous KOH, and vigorously aerated.

Similarly, aeration of suspensions of dihydro-fuscine (20 mg) in acetate buffer solutions (100 ml.) of either pH 5.0 or 6.0 failed to yield fuscine, both the buffer solutions and the undissolved crystals remaining colourless.

Analysis and general properties of fuscine

Fuscine was prepared for analysis by repeated recrystallization from ethanol and was finally obtained in brilliant orange diamond shaped plates, m.p. 230°. It sublimes in a high vacuum at 160–180° without decomposition. Found: C, 65.0, 64.9, H, 5.9, 5.7, N, S, Cl, OCH₃, nil, active hydrogen in pyridine, 0.38, in anisole, nil, C—CH₃, 10.2. C₁₈H₁₆O₈ requires C, 65.2, H, 5.8, 1 active hydrogen atom, 0.36, 2C—CH₃, 10.9%.

A molecular weight determination by the depression of the melting point of camphor failed owing to decomposition. Dr W. H. Taylor, Department of Physics, University of Manchester kindly carried out an X-ray analysis. He reported as follows: 'The unit cell of the crystal was found to be 1102, and the mol. wt. was calculated from this as 1102/2 = 551 or 1102/4 = 275. The symmetry of the crystal is such that it was not possible to decide between the two alternatives'. C₁₈H₁₆O₈ requires a mol. wt. of 276 and this value was confirmed by molecular weight determinations on derivatives of fuscine.

Fuscine is soluble in chloroform, acetone, hot ethanol, ethyl acetate, and acetic acid, moderately soluble in ether and benzene, slightly soluble in cold ethanol, and almost insoluble in light petroleum and water. It is readily soluble in aqueous alkalis (NH₃, NaHCO₃, Na₂CO₃ and NaOH) with the formation of an intense purple colour, and in aqueous pyridine with a purple blue colour. A 1% solution in chloroform showed no rotation in a 1 dm tube. An ethanolic solution of fuscine gives a purplish red colour with ethanolic FeCl₃. It is soluble in cold conc. H₂SO₄ with the formation of a greenish yellow, not very intense, colour. An ethanolic solution does not give a precipitate with Brady's reagent, nor does an acetone solution restore the colour to Schiff's reagent. With ethyl cyanoacetate in ethanolic NH₃ no blue

colour is developed, but only the purple colour which fuscine gives with any alkali. The purple colour changes to orange brown in the course of half an hour

Preparation of dihydrofusicin from fuscine

(a) *By reduction with sodium hyposulphite* A solution of fuscine (20 g) in chloroform (1800 ml.) was shaken vigorously with a solution of $\text{Na}_2\text{S}_2\text{O}_4$ (200 g) in water (1 l). When the original orange colour had disappeared completely the chloroform solution was washed once with a little freshly boiled out water and evaporated to dryness in an atmosphere of N_2 . The dry residue was crystallized from ethanol (120 ml) yielding dihydrofusicin (15.4 g) as colourless rhombic crystals, m.p. 206° (Found C, 64.3, 64.2, H, 6.6, 6.7 $\text{C}_{15}\text{H}_{18}\text{O}_5$ requires C, 64.7 H, 6.5%)

(b) *By catalytic reduction* A solution of fuscine (0.5 g) in glacial acetic acid (100 ml) was reduced by shaking in an atmosphere of H_2 with a catalyst prepared from PdCl_2 (0.2 g) and norite charcoal (0.5 g). Reduction was complete in 5 min when 45.4 ml. (corr.) of H_2 were absorbed. Theory for uptake of 1 mol $\text{H}_2 = 40.6$ ml. The colourless solution was separated from the catalyst by filtration and evaporated to dryness *in vacuo*. The residue, crystallized from dry ether, gave colourless, thick, irregular tablets, wt 0.30 g, m.p. 206° alone or in admixture with dihydrofusicin prepared by method (a) above. Adams & Shriner's (1923) platinum oxide catalyst similarly gave dihydrofusicin, no further reduction being effected.

Dihydrofusicin is moderately soluble in ethanol or ether and is almost insoluble in light petroleum or water. Its ethanolic solution gives a red colour with FeCl_3 .

Preparation of fuscine from dihydrofusicin

When suspensions of dihydrofusicin in buffer solutions of pH 8 or below are aerated, there is little evidence of oxidation. At pH 9 or above oxidation is rapid and appears to be accelerated by concentrations of FeSO_4 equal to those present in Czapek Dox solution. The optimum conditions are as follows. Dihydrofusicin (0.1 g) was suspended in 0.05M sodium borate buffer solution, pH 9.2 (10 ml), containing 0.1 ml of a 0.1% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ solution. The dihydrofusicin quickly dissolved with the formation of a dark purple solution which was vigorously aerated for 3 hr, and acidified with 2N HCl. The resulting orange flocculent precipitate (70 mg), on crystallization from ethanol, gave typical orange plates, m.p. 230° alone or in admixture with authentic fuscine.

Compound of fuscine with thioglycollic acid

Thioglycollic acid (0.5 ml. of 90%) was added to a solution of fuscine (0.24 g) in water (20 ml) and 0.1N NaOH

(50 ml). The initial purple colour disappeared at once and a pale yellow solution, pH 5.0, resulted. Four drops of 2N HCl were now added and colourless needles quickly began to crystallize. After chilling overnight they were collected, washed and dried, wt 0.29 g, m.p. 193° after shrinking at 120° . Recrystallization of 0.2 g from hot water (80 ml) yielded 0.13 g of colourless needles, m.p. 200° (Found (a) on different samples of crystals, m.p. 193° , C, 52.8, 52.8, H, 5.8, 5.2, S, 8.2, 9.2, (b) on crystals, m.p. 200° , C, 52.6, H, 5.2, S, 8.7 $\text{C}_{17}\text{H}_{22}\text{O}_5\text{S}$, i.e. $\text{C}_{15}\text{H}_{18}\text{O}_5 + \text{C}_2\text{H}_4\text{O}_2\text{S} + \text{H}_2\text{O}$, requires C, 52.8, H, 5.8, S, 8.3%) The compound is slightly soluble in cold and readily soluble in hot water. It also dissolves readily in 0.05M phosphate buffer, pH 8.0, to a colourless solution. When dissolved in an excess of 0.1N NaOH under conditions ensuring rigid exclusion of O_2 , a strong purple colour developed at once, but attempts to isolate fuscine, after acidification, failed. Much of the substance was recovered unchanged. It is remarkably stable towards acids. Thus 0.2 g suspended in 0.5N HCl (20 ml), was boiled under reflux for 6 hr in an atmosphere of N_2 . The solution remained colourless and 0.16 g of unchanged material, m.p. 200° , was recovered from the cooled solution.

SUMMARY

1 The preparation, from culture filtrates of two different strains of the microfungus *Oidiiodendron fuscum* Robak, of a hitherto unreported mould metabolic product *fusicin*, $\text{C}_{15}\text{H}_{18}\text{O}_5$, orange plates, m.p. 230° , is described.

2 It is accompanied in the culture filtrates by its reduction product *dihydrofusicin*, $\text{C}_{15}\text{H}_{18}\text{O}_5$, colourless rhombic crystals, m.p. 206° . These two compounds, which are readily interconvertible, account for the greater part of the antibacterial activity of culture filtrates of *O. fuscum*.

3 The general properties of fusicin and dihydrofusicin are described. Fusicin has many properties suggestive of a quinonoid structure, and dihydrofusicin those of the corresponding quinol.

4 Fusicin forms a stable crystalline compound with thioglycollic acid, having the molecular formula $\text{C}_{17}\text{H}_{22}\text{O}_5\text{S}$, i.e. $\text{C}_{15}\text{H}_{18}\text{O}_5 + \text{C}_2\text{H}_4\text{O}_2\text{S} + \text{H}_2\text{O}$.

The author offers his best thanks to The Therapeutic Research Corporation of Great Britain Ltd for a grant enabling him to carry out this work, and to Dr W. H. Taylor, Department of Physics, University of Manchester, for the determination of the molecular weight of fusicin by X ray analysis.

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Appendix Antibacterial Activity of Fusicin

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METHODS

A sterile 1/2500 solution of fusicin was prepared by dissolving fusicin (12 mg) in 0.1N NaOH (1 ml.) and diluting with sterile phosphate buffer (4 ml of pH 7.0) and sterile water (25 ml). The clear, intensely purple solution was mixed with an equal volume of sterile 2% glucose broth, the pH of the resulting solution being 7.6. Successive two fold dilutions were made in 2% glucose broth giving a range of concentrations of fusicin of 1/5000, 1/10,000, 1/20,000 up to 1/1,280,000. All the bacteria listed in Table 1 were tested on this medium except *Vibrio cholerae*, in which case the 2% glucose was omitted from the medium. The serial dilutions were quickly inoculated with 1 loopful of an undiluted 24 hr culture of the desired micro organism. Growth readings were taken after 24 hr incubation at 37° in all cases except *Corynebacterium diphtheriae* (48 hr) and the mycobacteria (72 hr).

A sterile solution of fusicin thioglycolic acid was prepared by dissolving 33 mg of the pure crystalline compound (see p. 531) in sterile water (50 ml.) and sterile 0.05M phos-

phate buffer (10 ml of pH 7.0). The resulting almost colourless solution, corresponding to a concentration of 1 in 2500 of uncombined fusicin, was used for making serial dilutions in sterile 2% glucose broth. These were inoculated with ten species or strains of micro organisms, incubated at 37° and growth readings were taken after 24 hr.

RESULTS

Fusicin The growth of all the eighteen species or strains of Gram positive bacteria tested was completely inhibited by fusicin, at concentrations varying from 1/80,000 to 1/1,280,000. Of the Gram negative bacteria it completely inhibited the growth of *Vibrio cholerae* at a concentration of 1/80,000, but six other species or strains were only inhibited at concentrations of 1/5000 to 1/10,000, and it was without appreciable effect on *Shigella flexneri* Type Z, *S. sonnei*, *Salmonella paratyphi* B and *Salmonella enteritidis* Gartner. It was completely active against

Table 1 Bacteriostatic power of fusicin against two species or strains of *Mycobacterium*, eleven of Gram negative and eighteen of Gram positive micro organisms

Micro organism	Catalogue no. (National Collection of Type Cultures or of London School of Hygiene and Tropical Medicine)	Limiting dilution for inhibition of growth	
		Complete	Partial
<i>Salmonella enteritidis</i> (Gärtner)	NCTC 125	—	5,000
<i>Salm. paratyphi</i> B	NCTC 14	—	5,000
<i>Shigella flexneri</i> Type Z	LSH	—	5,000
<i>S. sonnei</i>	LSH	—	5,000
<i>Escherichia coli</i>	LSH	5,000	10,000
<i>E. coli</i>	NCTC 86	5,000	—
<i>Salmonella typhi</i>	NCTC 2128	5,000	—
<i>Proteus vulgaris</i>	NCTC 5887	5,000	10,000
<i>Shigella shigae</i>	NCTC 4837	10,000	—
<i>Salmonella typhi murum</i>	NCTC 74	10,000	20,000
<i>Mycobacterium phlei</i>	NCTC 525	10,000	20,000
<i>Myc. smegmatis</i>	NCTC 523	20,000	—
<i>Vibrio cholerae</i>	NCTC 1633	80,000	160,000
<i>Bacillus subtilis</i>	LSH	80,000	160,000
<i>Staphylococcus aureus</i>	NCTC 4736	80,000	320,000
<i>Staph. aureus</i>	NCTC 3093	80,000	320,000
<i>Staph. albus</i>	NCTC 3256	80,000	320,000
<i>Corynebacterium diphtheriae gravis</i>	LSH	160,000	640,000
<i>Staphylococcus aureus</i>	NCTC 3750	320,000	—
<i>Staph. aureus</i>	NCTC 4163	320,000	640,000
<i>Staph. aureus</i>	LSH 2	320,000	640,000
<i>Bacillus anthracis</i>	NCTC 5444	320,000	640,000
<i>Corynebacterium diphtheriae intermedius</i>	LSH	320,000	640,000
<i>C. diphtheriae mitis</i>	LSH	320,000	1,280,000
<i>Staphylococcus aureus</i>	LSH 1	640,000	1,280,000
<i>Staph. aureus</i>	NCTC 3095	640,000	1,280,000
<i>Streptococcus viridans</i>	NCTC 3166	640,000	1,280,000
<i>Strep. pyogenes</i> Griffith Type 1	NCTC 2432	640,000	1,280,000
<i>Bacillus anthracis</i>	LSH	1,280,000	—
<i>Staphylococcus aureus</i>	NCTC 3761	1,280,000	—
<i>Staph. citreus</i>	NCTC 2301	1,280,000	—

Mycobacterium smegmatis at a concentration of 1 20,000 and *Myco phlei* at 1 10,000 but was completely inactive against an unidentified species of *Mycobacterium* not listed in Table 1

Fuscin thioglycollic acid The results obtained are summarized in Table 2 They indicate that, in general, the conversion of fuscin into its thioglycollic acid derivative caused a remarkably small change in its antibacterial spectrum, cf Table 1

SUMMARY

1 Fuscin is a somewhat powerful antibacterial substance, being particularly active against Gram-positive bacteria, but much less active or substantially inactive against mycobacteria and Gram negative bacteria

2 The conversion of fuscin into its thioglycollic acid derivative causes a remarkably small change in its antibacterial spectrum

Table 2 *Antibacterial activity of fuscin thioglycollic acid*

Micro organism	Catalogue no	Limiting dilution for inhibition of growth	
		Complete	Partial
<i>Escherichia coli</i>	NCTC 86	—	5,000
<i>Proteus vulgaris</i>	NCTC 5887	5,000	—
<i>Vibrio cholerae</i>	NCTC 1633	20,000	—
<i>Bacillus subtilis</i>	LSH	40,000	80,000
<i>Staphylococcus aureus</i>	NCTC 3093	40,000	160,000
<i>Staph aureus</i>	LSH 2	40,000	80,000
<i>Staph aureus</i>	Oxford H	160,000	320,000
<i>Staph aureus</i>	LSH 1	160,000	320,000
<i>Bacillus anthracis</i>	NCTC 5444	640,000	1,280,000
<i>Corynebacterium diphtheriae mitis</i>	LSH	640,000	1,280,000

Metabolism of Fluorene in the Rabbit

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The metabolism of the hydrocarbon fluorene does not appear to have been examined previously, although the compound has been investigated for carcinogenic activity in skin painting experiments with mice (Bloch, 1922, quoted by Kennaway, 1930, Twort & Fulton, 1930) and more recently in feeding experiments with rats (Wilson, De Eds & Cox, 1947). The results of these experiments led to the conclusion that fluorene is not carcinogenic. The high carcinogenic potency of 2 acetamidofluorene is now well established, as may be seen from the review by Bielschowsky (1947), but the only publication which has appeared to date concerning the metabolism of this carcinogen is that of Bielschowsky (1945) who showed that the metabolite, 7 hydroxy 2 acetamido fluorene, may be isolated from the urine of rats fed 2 acetamidofluorene.

In the course of further studies of the metabolism of 2 acetamidofluorene in rabbits an investigation was made concerning the fate of fluorene in these animals with the primary object of determining whether the reactive 9 methylene group of this compound could undergo any change during the passage

of the hydrocarbon through the animal body. This work might lead to a better insight into the metabolism of the related amine carcinogen. It was also of interest to see if fluorene is metabolized by rabbits according to the hydroxylation mechanism which has been found to play a part in the detoxication of a number of carcinogenic and non carcinogenic polycyclic hydrocarbons. Adequate reviews of this detoxication mechanism have been given by Boyland & Weigert (1947) and by Williams (1947), only one example will be quoted here. Young (1947) found that the urine from rats ingesting naphthalene contained the diol, 1 2 dihydroxy-1 2 dihydronaphthalene, which yielded α naphthol on mild acid hydrolysis, and Bourne & Young (1933) showed that naphthalene in man and dog gave rise to α naphthol and the glucuronide of this compound. These results are in accord with the views of Fieser (1941) that the detoxication reaction involves primarily the addition of the elements of hydrogen peroxide to the hydrocarbon molecule in a perhydroxylation reaction giving rise to diols which are usually unstable and which pass over into phenolic compounds by

loss of water from the diol grouping. With regard to the positions in the hydrocarbon nucleus at which *in vivo* hydroxylation occurs the interesting observation was made by Berenblum & Schoental (1943) that benzanthrane and dibenzanthracene are metabolically attacked (hydroxylated) at points where sulphonation of these hydrocarbons occurs *in vitro*, and Bielschowsky showed that this rule applied to 2 acetamidofluorene which is metabolized by rats to the 7-hydroxy compound, 2 aminofluorene being sulphonated *in vitro* at position 7 according to Courtot (1930).

Although no evidence has been found that the reactive methylene grouping of fluorene is attacked *in vivo* it has been shown that 2 hydroxyfluorene and a compound which analyzes correctly as the glucuronide of this phenol can be isolated from the urine of rabbits ingesting fluorene.

EXPERIMENTAL

Materials and methods The fluorene sample employed in this work was obtained by crystallizing a commercial fluorene preparation from ethanol. It melted at 113°. For an experiment in which 2-hydroxyfluorene was fed to a rabbit, this phenol (m p = 168°) was prepared from 2 amino fluorene according to the method of Diels (1901).

The detection and identification of fluorene derivatives occurring in the urine of rabbits ingesting fluorene compounds was facilitated by means of sensitive colour reactions given by traces of fluorene derivatives when these are treated with drops of a solution of *p* dimethyl aminobenzaldehyde (5% w/v) in conc H_2SO_4 . These colour reactions are described in the text and the use of the reaction will be discussed more fully in another communication. All melting points are uncorrected. Carbon and hydrogen analyses were carried out by Drs Weiler and Strauss (Oxford).

RESULTS

Tolerance of the rabbit to fluorene A female rabbit which had not been used for any other experiment was maintained on a stock diet consisting of oats (1 part), sharps (1 part), bran (2 parts) and milk powder (2 parts). The animal received 70 g/day of this diet in which was incorporated 250 mg of fluorene, the diet being mixed thoroughly with the addition of water. 100 g of cabbage was fed daily. The animal received 1.5 g of fluorene (at 250 mg/day) and 10 days after this experiment it received 1 g of fluorene at the same dosage level/day. The animal gained in weight over the period of the experiment.

In another experiment in which a male rabbit (initial weight, 2120 g) was fed fluorene at the level of 250 mg/day in 70 g of stock diet for 20 consecutive days an increase in body weight of 400 g was observed over this period. The animal appeared to be in good health and 2 months after the end of the

experiment it weighed 2450 g. It would appear then that rabbits tolerate fluorene well and that ingestion of the compound for short periods at least has no serious effect on the health of the animals.

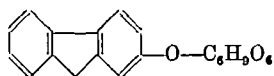
The animals receiving fluorene were kept in metabolism cages, the urine being collected daily and preserved with chloroform in a refrigerator. The urine contained appreciable amounts of phosphate and gave a positive Tollens test for glucuronic acid. When several drops of the urine were placed in a depression in a glazed white tile and treated with 0.25 ml of *p* dimethylaminobenzaldehyde reagent a bright purple pink coloration developed which soon changed to green on standing. This colour reaction indicates the presence of fluorene derivatives, normal rabbit urine giving no purple colour with the reagent.

Isolation of 2-hydroxyfluorene In an experiment in which 1.75 g of fluorene was fed to a rabbit at the rate of 250 mg/day, 900 ml of urine were collected. The urine, which was alkaline, was divided into three portions of 300 ml, each of which was extracted five times with 100 ml of ether. To obtain a satisfactory separation, the ether urine emulsions were centrifuged and the combined ether extracts were dried over anhydrous Na_2SO_4 . The ether extract was concentrated to 10 ml, poured into a small flask containing about 15 ml of water and the flask warmed carefully until all the ether had been expelled. Solid material separated giving a purple coloration when treated with a drop of 5% (w/v) *p* dimethylaminobenzaldehyde in concentrated sulphuric acid. The same colour reaction was given by synthetic 2 hydroxyfluorene. The solid product was filtered and dried at the pump. It was then shaken thoroughly with cold dilute caustic soda solution to dissolve as much of the product as possible, and filtered. The dark coloured filtrate was acidified with concentrated hydrochloric acid and the resulting crude brown precipitate was washed with water and dried at 37°. This product (100 mg) was crystallized several times from water in the presence of activated charcoal, and a white substance (40 mg) was obtained which, after drying, had m p 167–168°, and mixed m p 168° with a sample of synthetic 2 hydroxyfluorene (Found C, 85.53, H, 5.64. Calc for $C_{13}H_{10}O$ C, 85.67, H, 5.55%).

About 30 mg of biosynthetic 2 hydroxyfluorene was dissolved in dilute NaOH and the solution treated with a drop of dimethyl sulphate. The white precipitate which appeared after a time was collected and crystallized from ethanol/water. It melted at 100–104°, mixed m p 104° with 2 methoxyfluorene m p 104° (Werner, 1902, gives 106–108°), prepared by treating synthetic 2 hydroxyfluorene with dimethyl sulphate. Both specimens of methoxy compound gave the same ink blue coloration when traces

were treated with a drop of *p* dimethylamino benzaldehyde reagent. The colour developed is quite distinct from the purple colour given by 2 hydroxyfluorene in this test. Pure samples of 2 hydroxyfluorene and 2 methoxyfluorene gave no colour reaction with concentrated H_2SO_4 only.

Isolation of a glucuronide The urine which had been freed from 2 hydroxyfluorene by ether extraction was acidified with concentrated hydrochloric acid, about 20 ml of acid being added to 300 ml of urine (slightly more acid than is required to make the urine acid to Congo red). Ether extraction was performed as described for the alkaline urine, but the extraction was incomplete since ether soluble orange coloured material could still be obtained by further extraction of the urine with ether. The ethereal extract was orange red in colour and after removal of the ether a dark red oil was obtained. A small amount of this oil gave a good Tollens reaction for glucuronic acid. The red oil was suspended in about 10 ml of water and the suspension boiled in the presence of activated charcoal. On cooling the filtrate, white needles separated. After two more crystallizations of this material from water, the colourless filtrate yielded fine long white needles on cooling. This product, which gave a positive reaction for glucuronic acid, was dried at 37° overnight. It melted at 214–215°. The compound analyzed as the *dihydrate* of *fluorene 2 glucuronide*.



(Found C, 58.1, H, 5.57 $\text{C}_{19}\text{H}_{18}\text{O}_7 \cdot 2\text{H}_2\text{O}$ requires C, 57.84, H, 5.63%) After drying *in vacuo* at 100°, anhydrous *fluorene 2 glucuronide* was obtained (Found C, 62.36, H, 5.46 $\text{C}_{19}\text{H}_{18}\text{O}_7$ requires C, 63.66, H, 5.07%). When traces of the material were treated with a drop of concentrated sulphuric acid the crystals became dark green, and after mixing with a glass rod black particles were observed in a pale green solution. With a drop of *p* dimethyl aminobenzaldehyde reagent the crystals became bright purple blue (reminiscent of the purple solution given by 2 hydroxyfluorene with the aldehyde reagent) finally yielding black particles in pale purple sulphuric acid medium.

Continuous extraction of acid urine Very satisfactory yields of the glucuronide were obtained when 300 ml of acidified urine (previously extracted when alkaline to remove 2 hydroxyfluorene) were subjected to continuous extraction with ether in a Kutscher Stoudel apparatus. After several hours, long white needles separated on the walls of the extraction flask just above the level of the boiling ether, and within 15–20 hr, quite a large amount of fairly pure glucuronide was obtained. The product was removed from the flask and after one crystal-

lization from water and drying overnight at 37° was obtained as fine long white needles melting at 214–215°. The yield of pure compound was 100 mg. It was sparingly soluble in ether, insoluble in benzene, slightly soluble in ethanol, and quite soluble in dilute sodium carbonate. On drying *in vacuo* at 100° to constant weight, 3.908 mg glucuronide lost 0.255 mg (6.5% H_2O). Theoretical loss for $\text{1H}_2\text{O}$, 4.8%, for $2\text{H}_2\text{O}$, 9.1%. The compound analyzed as a *hydrate of fluorene-2 glucuronide* (Found C, 59.92, H, 5.92 $\text{C}_{19}\text{H}_{18}\text{O}_7 \cdot 1\frac{1}{2}\text{H}_2\text{O}$ requires C, 59.1, H, 5.45%). After drying to constant weight *in vacuo* at 100°, *fluorene-2 glucuronide* was obtained (Found C, 63.62, H, 5.24 $\text{C}_{19}\text{H}_{18}\text{O}_7$ requires C, 63.66, H, 5.07%). The compound appears to have a low specific rotation. 5 ml of a solution of the glucuronide dihydrate (5 mg/ml), prepared by dissolving the glucuronide in a solution of sodium carbonate containing sufficient alkali to form the monosodium salt, gave in a 1 dm tube a very small laevorotation (–0.05°). There can be little doubt that the compound is the dihydrate of the glucuronide of an hydroxyfluorene which is thought to be the 2 hydroxy compound. Unsuccessful attempts have been made to isolate this phenol from the products of acid hydrolysis of the glucuronide. It is thought that hydrolytic products of the glucuronide are condensing with hydroxyfluorene at the 9 methylene group thus preventing isolation of the hydroxyfluorene.

A note on the metabolism of 2 hydroxyfluorene in the rabbit

In the hope of obtaining a glucuronide of 2 hydroxyfluorene for comparison with the glucuronide isolated from the urine of rabbits ingesting fluorene, it was decided to examine the metabolism of 2 hydroxyfluorene fed at the level of 250 mg/day in 70 g stock diet to a male rabbit. The animal tolerated the material well and in the course of 10 days, during which time it ingested 2.5 g of 2 hydroxyfluorene, it steadily gained in weight from 1670 to 1840 g. Five days after the experiment was terminated the rabbit weighed 1980 g. The urine from this animal gave a positive Tollens reaction and when 0.25 ml of urine was treated with 0.25 ml of *p* dimethyl aminobenzaldehyde reagent the bright purple colour characteristic of 2 hydroxyfluorene was obtained. With conc H_2SO_4 only the urine gave no coloration. 2 Hydroxyfluorene could be isolated readily from the alkaline urine by ether extraction, but no solid glucuronide separated from the acidified urine (20 ml conc hydrochloric acid/300 ml urine) submitted to continuous extraction with ether. The ether extract, however, gave a positive reaction for glucuronic acid. It would appear that 2 hydroxyfluorene is excreted for the most part unchanged. Certainly

there is very little conjugation with glucuronic acid. The urine was not examined for the presence of ethereal sulphates

A note on the occurrence of fluorene in faeces

About 40 g of faeces were collected from a rabbit which was receiving 250 mg fluorene/day. The faeces were allowed to dry at room temperature for several days and then ground to a powder with an equal weight of anhydrous sodium sulphate. The powder was ground under ether repeatedly and after each extraction the ether was filtered off, the combined ethereal extracts concentrated, dried over anhydrous sodium sulphate and evaporated to dryness. The dark green residue was taken up in light petroleum (b.p. 40–60°) and chromatographed on activated alumina (British Drug Houses product). All pigments remained on the column and the petroleum ether eluate was colourless. A drop of this eluate was allowed to evaporate on a glazed white tile and the residue treated with a drop of *p* dimethylamino-benzaldehyde reagent. A bright pink colour very like that given by fluorene developed. No colour appeared when a similar experiment was carried out using instead a drop of concentrated sulphuric acid. Several other experiments were carried out with faeces collected at different times and whilst the light petroleum extract sometimes gave a positive reaction for fluorene, on other occasions it did not. It is concluded that little if any fluorene is excreted in the faeces, the positive reaction for fluorene sometimes observed being due probably to contamination of the faeces with the hydrocarbon from the food. The reaction for fluorene is extremely sensitive, as little as 1 µg may be detected readily.

DISCUSSION

The metabolites obtained from the urine of rabbits ingesting fluorene are 2-hydroxyfluorene and the glucuronide of this phenol. Data relating to the yields of these metabolites were accidentally destroyed so that only a rough estimate of the recoveries can be made. In the urine collected from a rabbit which had ingested 1.75 g of fluorene about 40 mg of pure 2-hydroxyfluorene was obtained, and by continuous extraction with ether the acidified urine yielded approximately 400–500 mg of pure glucuronide (computed from recoveries obtained from fractions of the total urine). Both metabolites were isolated in the above proportions from the separate urines of two rabbits ingesting fluorene. Little if any un-

changed fluorene is excreted in the faeces and this appears to be in agreement with the work of Chang (1943), who showed that only small amounts of the simple hydrocarbons such as naphthalene and phenanthrene are eliminated in the faeces of rats receiving these compounds, whereas considerable amounts of the more complex hydrocarbons such as methylcholanthrene are excreted by this route.

The metabolism of the hydrocarbon follows the rule stated by Berenblum & Schoental (1943) (and confirmed by Bielschowsky (1945) for the case of 2-acetamidofluorene) that the site of biological hydroxylation is that at which sulphonation occurs *in vitro*. Courtot & Geoffroy (1924) showed that fluorene-2-sulphonic acid is the first product of sulphonation of the hydrocarbon *in vitro*.

It is possible that 2-hydroxyfluorene arose from diols during the isolation of the compound from the urine, although the crude solid extract after decolorization by charcoal was found to be optically inactive, whereas diols of fluorene, if present, would be expected to show optical activity.

With regard to the tolerance of the rabbit to fluorene it is interesting to note that Wilson *et al* (1947) found that groups of rats maintained on a diet containing fluorene at the levels of 0.062–0.5% for 106 days were in good health after 453 days and at autopsy they showed no signs of gross tissue damage. The growth rate of rats receiving fluorene at concentrations of 0.5–1% was impaired, but the health of these animals appeared to be good.

SUMMARY

1 Two metabolites of fluorene have been isolated from the urine of rabbits, viz. 2-hydroxyfluorene and a compound which analyzes correctly for the glucuronide of this phenol. This is a further example of the biological hydroxylation of a hydrocarbon taking place at the point at which sulphonation occurs *in vitro*.

2 Little if any fluorene is eliminated in the faeces and no metabolites of the hydrocarbon have been detected in these.

3 2-Hydroxyfluorene fed to a rabbit appears to be excreted largely unchanged.

4 Fluorene and 2-hydroxyfluorene are well tolerated by the rabbit.

I wish to express my best thanks to Prof. H. N. Green for his interest in this work and to Mr. J. Westrop for carrying out some of the experimental work. The work was supported by a grant from the British Empire Cancer Campaign.

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Determination of the Arginase Activities of Homogenates of Liver and Mammary Gland Effects of pH and Substrate Concentration and Especially of Activation by Divalent Metal Ions

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In our previous studies of the arginase activities of certain tissues of the rat (Folley & Greenbaum, 1946, 1947*a*) we used reaction periods of 30 min and 20 hr for homogenates of liver and mammary gland respectively, and assessed the activities of the homogenates in terms of our 30 min or 20 hr arginase units from enzyme calibration curves. Arginase undergoes inactivation at alkaline pH values (Hunter & Morrell, 1933*a*), a factor particularly serious in methods involving long reaction periods. Nevertheless, we feel confident that our previous findings regarding changes in the arginase activity of the mammary gland under various conditions are valid, since all values were read from a calibration curve which not only was reproducible with different mammary gland homogenates, but also agreed with a curve obtained for one of these homogenates in the presence of Co^{++} (see Fig. 1). The significance of the latter agreement is not lessened by our present belief (see later section) that the effect of Co^{++} under these conditions was mainly to inhibit reversible inactivation due to dissociation of the enzyme system.

However, the use of different units for liver and mammary gland homogenates was hardly practicable for further studies involving comparison of arginase activities of these tissues in the same animal, since the comparison can only be made if the quantitative relationship between the two units is known, and while the highly active liver homogenates can be assayed by both methods, the high dilution necessary for the 20 hr method favours dissociation of the

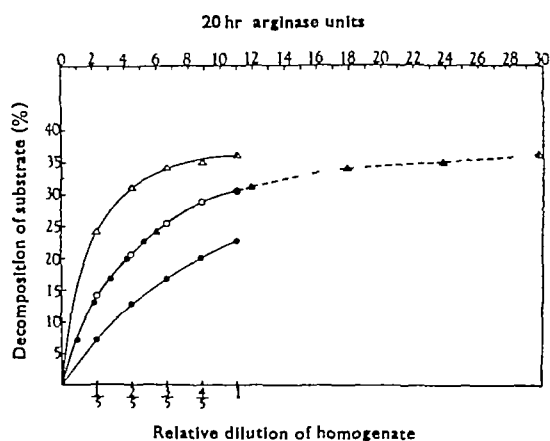


Fig. 1 Calibration curve (circles) for the determination of arginase in mammary gland homogenates by the method of Folley & Greenbaum (1947*a*). The bottom curve (black dots) represents the arginase activities of various dilutions of an homogenate less active than the one used for the construction of the calibration curve, the top curve (triangles) represents the arginase activities of corresponding dilutions of the same homogenate in the presence of Co^{++} . The upper horizontal scale (20 hr arginase units) refers only to the central curve, to which the other two sets of points have been fitted by appropriate adjustment of their horizontal scales, i.e. transformation of their abscissae into 20 hr arginase units. It will be seen that the bottom curve gives a very satisfactory fit to the standard calibration curve, while the greater part of the top curve provides an extension (broken line) of it.

metal enzyme with consequent loss of activity at the outset. In addition, we wished to investigate the activation of the arginase in mammary-gland and liver homogenates by certain divalent metal ions. Here again, methods involving prolonged hydrolysis are unsuitable, since, if the supposed activating agent merely protects the enzyme against inactivation, it will apparently increase its activity. For these two purposes we require, therefore, a method applicable to homogenates with widely different activities, yet employing so short a reaction period that approximate initial velocities are measured.

The sensitive colorimetric reagent for urea described by Archibald (1945), and applied by Van Slyke & Archibald (1946) to the determination of arginase activity, was the best basis for a method allowing the estimation of approximate initial reaction velocities for tissues with such widely different activities as liver and mammary gland. The advantages of a colorimetric method for carrying out a number of determinations simultaneously are obvious, and it is better to determine liberated urea rather than residual arginine, not only because of the remote possibility that arginine may be decomposed by enzymes other than arginase without the liberation of urea (see Van Slyke & Archibald, 1946), but also because the relative changes in arginine, for short reaction periods, are very small. Whilst we have no reason to believe that our earlier experiments (Folley & Greenbaum, 1947*a*), in which considerable proportions of the substrate were hydrolyzed, were affected by errors due to either of these factors, we have now adapted the photometric method of Van Slyke & Archibald (1946) for use with our tissue homogenates.

The pH-activity and substrate concentration-activity relationships under our conditions have been determined, and also the activation of the arginase of homogenates by Mn^{++} and Co^{++} . The results show that Mn^{++} is a better activator than Co^{++} and that the enzyme exists in the tissues as an easily, but reversibly, dissociable manganese protein complex which may not always be fully activated.

EXPERIMENTAL

Tissue homogenates Homogenates of liver and of lactating mammary gland of rats were prepared as described previously (Folley & Greenbaum, 1947*a*) with the homogenizer of Folley & Watson (1948). Tissues were homogenized for 5 min at room temperature, liver with 9 vol of saline (0.9% NaCl) and mammary gland with 19 vol, the larger dilution being necessitated by the limited amount of gland usually available and the dimensions of the homogenizer. The amount of mammary tissue available from one rat is restricted since only the 'abdominal' (i.e. the two abdominal and four inguinal) glands, and not the thoracic glands, which are much more richly interlaced with strands of muscle and connective tissue, were used. The tissues were dissected out and homogenized as rapidly as possible, and the enzyme determinations always started within 15 min of the death of the rat, which was killed by dislocation of the spine.

In much of this work it was found convenient to carry out activity determinations on liver homogenates further

diluted 1:20 with saline (1 ml \equiv 5 mg fresh tissue), whilst mammary gland homogenates were used undiluted (1 ml \equiv 50 mg fresh tissue). For activity determinations involving only 1 min hydrolysis, liver homogenates were further diluted only 1:8 (1 ml \equiv 12.5 mg fresh tissue).

Preparation of reagents The urea reagent of Archibald (1945) (α -isonitrosopropiophenone) was synthesized by the method of Clausen & Manasse (1889). Yield, c. 75%, m.p. after recrystallization from hot water, 114–115° (uncorr.). The substrate used throughout was L-arginine monohydrochloride, prepared from gelatin by the method (1) of Brand & Sandberg (1932).

Determination of enzyme activity The method finally adopted, on the basis of the present results, for routine determination of the arginase activity of tissue homogenates differs from that of Van Slyke & Archibald (1946) mainly in that we use a higher reaction temperature (37°) and a lower substrate concentration (0.227M), the latter being just sufficient to give the maximum initial velocity at our (optimal) pH of 9.45.

The reaction mixture consists of 2.5 ml of 8.0% (w/v) L-arginine monohydrochloride (titrated to pH 9.45 at 37°), 1.25 ml of glycine buffer (pH 9.45 at 37°) and 0.3 ml of a suitably diluted homogenate. The buffer substrate is preheated to 37° in the thermostat, whilst the homogenate, which is best kept at room temperature if it contains no added Mn^{++} , is diluted if necessary to the required degree with saline at 37° immediately before mixing with the buffer substrate. The reaction is stopped after a given time (usually 5 min) by the addition of 0.95 ml of freshly prepared 30% (w/v) HPO_3 , and the precipitate of protein filtered off after 30 min at room temperature. Thereafter the colour is developed as described by Van Slyke & Archibald (1946), 2.0 ml of filtrate being used and the colour measured in a Miller photoelectric absorptiometer (Ilford spectrum green filter no. 604), usually with a 1 cm cell or, with intense colours, a 0.5 cm cell. An enzyme blank is set up and treated in exactly the same way, except that the HPO_3 is added before the homogenate. The arginase unit is now defined as that quantity of enzyme which, under the conditions specified above, liberates 1 μ mol of urea (0.06 mg)/min at 37° and pH 9.45 from L-arginine in 0.227M concentration.

If the fully activated arginase activity is required, sufficient $MnSO_4$ solution to give a final concentration of 2 mg Mn^{++} /ml of homogenate is pipetted into a test tube and taken to dryness. The requisite amount of homogenate, if necessary (e.g. with liver homogenates) diluted ready for enzyme determination, is then added and incubated at 37° for 1 hr. These conditions have been found to give full activation (see later section). Alternatively, the tissue can be homogenized with saline containing the correct concentration of Mn^{++} , and any subsequent necessary dilution carried out with saline containing 2 mg Mn^{++} /ml. In the presence of Mn^{++} (or Co^{++}) under these conditions, a fine flocculent precipitate is formed in the homogenate, removal of which would almost certainly result in loss of enzyme activity (see, e.g., Hunter & Downs, 1944). This precipitate can be uniformly dispersed by vigorous shaking just before pipetting.

We recommend a 5 min hydrolysis period for routine use, except for relatively inactive homogenates, though in the present work we have often used 10 min. Periods longer than 10 min are neither necessary with this technique, nor desirable, if inactivation of the enzyme during the run is to

be minimized. The method is however sufficiently sensitive to allow further reduction to 1 min with liver homogenates if desired, however, beyond this point random errors in measurement of time assume undue importance.

Investigations described later show that with reaction periods of either 5 or 10 min, if more than about 1% of the substrate is hydrolyzed, the observed urea production is no longer linearly related to the enzyme concentration and an enzyme calibration curve must be used, whereas below this limit the arginase units can be arithmetically calculated. The reaction period (max. 10 min) and the dilution of the homogenate should therefore be chosen accordingly. It is recommended that for 5 min reaction periods no more than 300 units of enzyme/l ($\approx 0.66\%$ hydrolysis in 5 min) be taken.

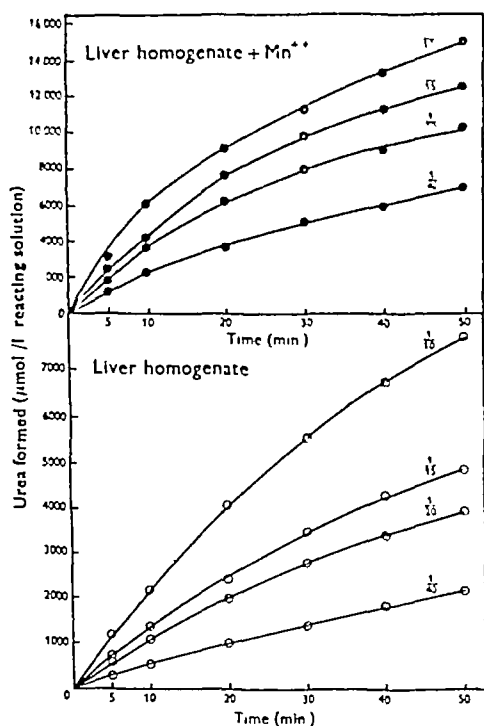


Fig 2 Arginase reaction velocity curves for various dilutions of a rat-liver homogenate, with and without added Mn^{++} . The reaction conditions were as follows: temperature 37° , pH 9.45, substrate concentration 0.227 M. The dilution of the original homogenate used for each experiment is indicated near the relevant curve.

According to Van Slyke & Archibald (1946) arginine diminishes the colour arising from the reaction between urea and α -nitrosopropiophenone, and thus we have confirmed. It is accordingly necessary to correct for this and any interference with the colour development which may be due to other components of the hydrolysates. Accordingly, galvanometer readings are converted into mg urea by reference to a standard curve constructed from measurements on a series of mock hydrolysates containing known amounts of urea and appropriate amounts of all other components. Correction for the possible interfering effects of substances present in the tissue homogenates is obviously impracticable,

and such effects are almost certainly negligible. Separate standardization curves for experiments in which Mn^{++} (or Co^{++}) are added to the homogenates are necessary (Mn^{++} intensifies the colour), and were constructed in a similar way. All our galvanometer curves were linear.

Van Slyke & Archibald (1946) imply that the pH (9.5), which they recommend for routine arginase activity determinations is slightly on the acid side of the optimum for liver enzyme, and they give reasons why they prefer to work at this pH rather than at pH 9.7, at which, they state, arginase may be slightly more active. The pH we specify for

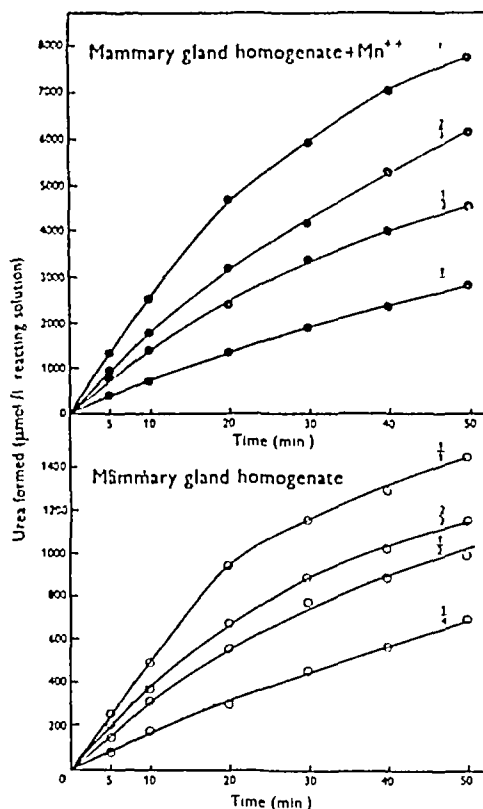


Fig 3 Arginase reaction velocity curves for various dilutions of a rat mammary gland homogenate with and without added Mn^{++} . The reaction conditions were as follows: temperature 37° , pH 9.45, substrate concentration 0.227 M. The dilution of the original homogenate used for each experiment is indicated near the relevant curve.

routine determinations is slightly more acid than that used by Van Slyke & Archibald, but is, nevertheless, the optimum for the enzyme in both liver and mammary gland homogenates. However, the increased stability of the enzyme on the acid side of the optimum (Hunter & Morrell, 1933a) made it desirable to carry out parallel studies at lower pH, particularly since a number of authors (Hunter & Dauphinee, 1930, Lightbody, 1938, Kochakian, 1944, Hunter & Downs, 1944, Folley & Greenbaum 1946, 1947a) have described arginase activity methods involving hydrolysis on the acid side of the pH optimum. For activity determinations at pH 9.1, the lower pH adopted, we used

substrate in 0.071M final concentration, which our investigations (see later section) indicated as the optimal concentration at this pH.

Throughout, in investigating the effect of changes in various conditions on arginase activity, the hydrolyses were carried out basically as above, but with appropriate modifications according to the factor under study.

RESULTS AND DISCUSSION

Velocity of arginase action

Reaction-velocity curves, determined at pH 9.45 and in presence of 0.227M substrate with and without added Mn^{++} , for various dilutions of rat liver and mammary-gland homogenates respectively, are given in Figs 2 and 3. In neither case does any

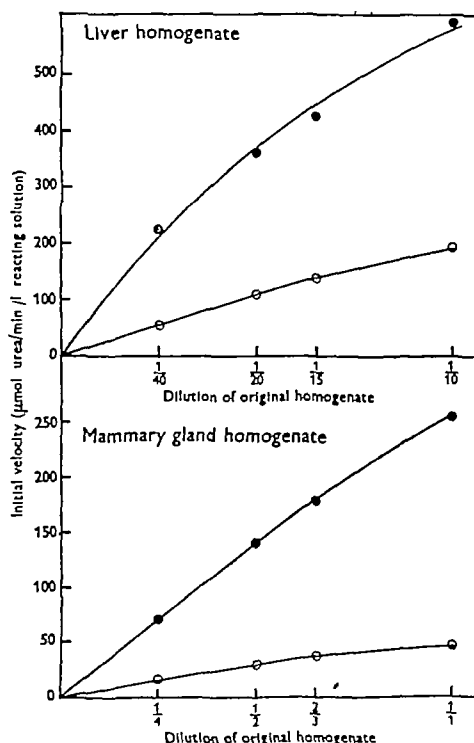


Fig 4 Relation between arginase concentration and initial reaction velocity, calculated from the urea production in the first 10 min, for homogenates of rat liver and mammary gland, with (black dots) and without (circles) added Mn^{++} . All experiments at 37° and pH 9.45, substrate concentration 0.227M.

reaction rate depart significantly from linearity over the first 10 min, though inhibitory effects, probably due to progressive inactivation of the enzyme and to the accumulation of the inhibitory reaction product L ornithine (Edlbacher & Zeller, 1936), become apparent soon after. In the runs with activated enzyme any progressive inactivation which occurred may be assumed to be due to the high alkalinity (see

Hunter & Morrell, 1933a) and was presumably irreversible, the non-activated enzyme would also be liable to reversible inactivation due to gradual dissociation of the metal-enzyme (see later section).

Thus for reaction periods up to about 10 min the amount of urea produced in a given time is proportional to the initial reaction velocity. Since the enzyme was in the presence of excess substrate, it may be anticipated that initial reaction velocities will be proportional to enzyme concentrations, so that for reaction periods up to 10 min the amounts of urea produced should be convertible by simple calculation into arginase units without reference to an enzyme calibration curve, as was found by Van Slyke & Archibald (1946) under their conditions. This is indeed the case, provided no more than about 10% of the initial substrate is hydrolyzed (Fig 4), the only curve which deviates appreciably from linearity is that for Mn^{++} -activated liver homogenate. Similar results were obtained at pH 9.1 in presence of 0.071M substrate, though in this case the upper limit of substrate decomposition for preservation of an approximately linear relation between enzyme concentration and urea production was 5%.

pH-Reaction velocity relationships

In investigating pH activity relationships the enzyme activities were determined by the standard method except that appropriate glycine buffers were used, and the substrate solutions titrated beforehand to the requisite pH. For each pH value an extra reaction tube was provided solely for pH determination at the beginning and end of the hydrolysis. In no case was any appreciable change in pH observed during the reaction. All pH values were measured by glass electrode at 37°.

Typical curves for homogenates of liver and mammary gland from the same rat, determined in presence of 0.227M substrate, are shown in Figs 5 and 6, the ordinates in Fig 6 representing initial reaction velocities expressed as percentages of the maximum. These curves cover a narrower pH range (particularly on the acid side of the optimum), but with smaller intervals than those commonly given in the literature so that they allow of closer definition of the pH optima than usual.

The pH optima (substrate concentration 0.227M) for non activated homogenates of both tissues were essentially the same (pH 9.43–9.45). Similar optima for liver arginase were reported by Felix & Schneider (1938) and for both liver and jack bean enzyme by Damodaran & Narayanan (1940). For liver arginase, more alkaline optima were given by Edlbacher & Bonem (1925) (pH 9.5–9.8), Hunter & Morrell (1933b) (pH 9.8) and Mohamed & Greenberg (1945) (pH 10.0), and more acid optima by Hino (1926) (pH 7.3–7.5), Edlbacher & Simons (1927) (pH 9.0) and Hellerman & Stock (1938) (pH 8.0). The latter

value may be compared with the value pH 9.0 given by Stock, Perkins & Hellerman (1938) for jack bean arginase. Most of the above mentioned studies were concerned with glycerol extracts of liver or with partially purified liver extracts, but differences among these results, and between many of them and our own, may result from the dependence of apparent pH optima on many factors, hence there seems no reason to conclude that the pH activity relationship for liver and mammary gland homogenates differs in any important respect from that of purified preparations.

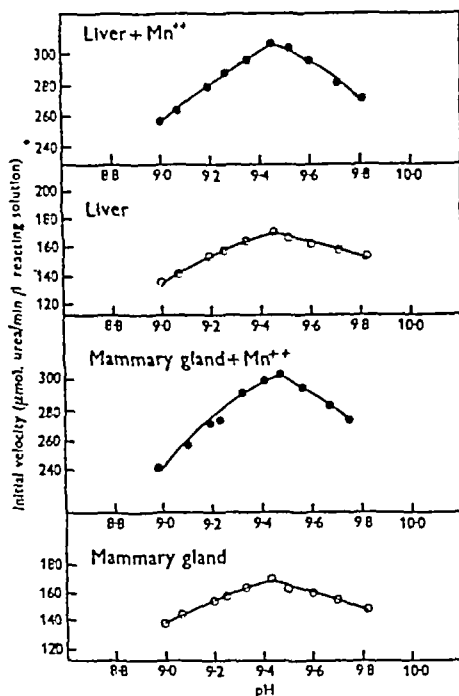


Fig 5 Activity pH curves for arginase in homogenates of rat liver and mammary gland, with (black dots) and without (circles) added Mn^{++} . All experiments at 37° and in presence of 0.227 M substrate. Reaction time 5 min.

Under our conditions, the pH optimum was unchanged by maximal activation with Mn^{++} (Figs 5 and 6), and in fact, with the method of plotting used in Fig 6, the points corresponding to activated and non activated homogenates of both tissues fall on the same curve. In this respect tissue homogenates appear to differ from liver extracts or crude extracts of jack bean, for appreciable shifts of the pH optimum to more acid values in the presence of activating divalent cations have been reported by Hellerman & Stock (1938), Stock *et al* (1938), Damodaran & Narayanan (1940) and Mohamed & Greenberg (1945), whilst Hellerman & Stock (1938) found that, though Ni^{++} and Co^{++} moved the optimum in the acid direction, Mn^{++} had the

opposite effect. It is tempting, but perhaps unjustifiable at this stage, to conclude that since activation with Mn^{++} does not affect the pH optimum the behaviour of the enzyme in our homogenates is more 'natural' than that in purified enzyme preparations. In any event, the fact that full activation causes no appreciable change in pH optimum under our conditions facilitates the development of an arginase assay applicable to activated and non activated homogenates alike.

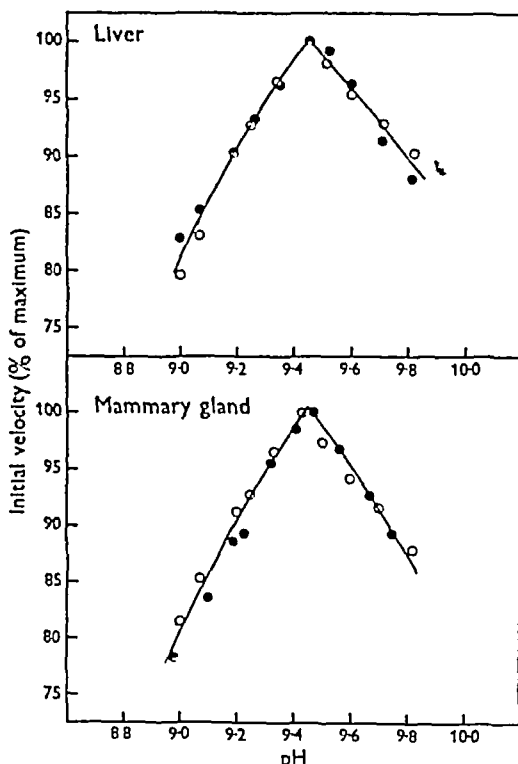


Fig 6 Activity pH curves for arginase in homogenates of rat liver and mammary gland, with (black dots) and without (circles) added Mn^{++} . All experiments at 37° in presence of 0.227 M substrate. Reaction time 5 min. The ordinates represent initial reaction velocities expressed as percentages of the initial velocity at the optimum pH.

The pH activity relationships in the presence of 0.071 M substrate are shown in Fig 7, in which the ordinates represent percentages of the maximum reaction velocity. With mammary-gland homogenates activation with Mn^{++} again had no effect on the pH optimum which, moreover, was unaffected by the change in substrate concentration. The pH optimum for the non activated liver homogenate was now, however, slightly more acid (approx pH 9.35), so that there is some indication in this case of a slight shift of the optimum (0.1 pH) towards the alkaline side in the presence of Mn^{++} .

The fact that an approximately threefold decrease in the substrate concentration had no appreciable effect on the pH optima is somewhat surprising, since there is evidence that K_m for arginase changes with pH (Greenberg & Mohamed, 1945). As will be shown in the next section, the lower of the two substrate concentrations used is optimal at pH 9.1 (inhibition occurring as the substrate concentration

The pH activity relationships for the arginase in tissue homogenates, at any rate in presence of excess substrate, are fairly critical. Thus at pH 9.1 and in presence of 0.227 M substrate, the velocity is only about 85% of that at pH 9.45. Since the curvature of pH-activity curves may well vary among homogenates from different animals, or homogenates from different tissues of the same animal (e.g. Fig. 7), tissue arginase assays for purposes of quantitative comparison should be carried out at the optimal pH and with adequate buffering to prevent pH changes during the reaction. Furthermore, methods which involve hydrolysis on the acid side of the pH optimum should be used with caution.

Substrate concentration reaction velocity relationships

Reaction velocity substrate concentration curves, determined at pH 9.45, are shown in Fig. 8. In these experiments it was necessary to correct for variable

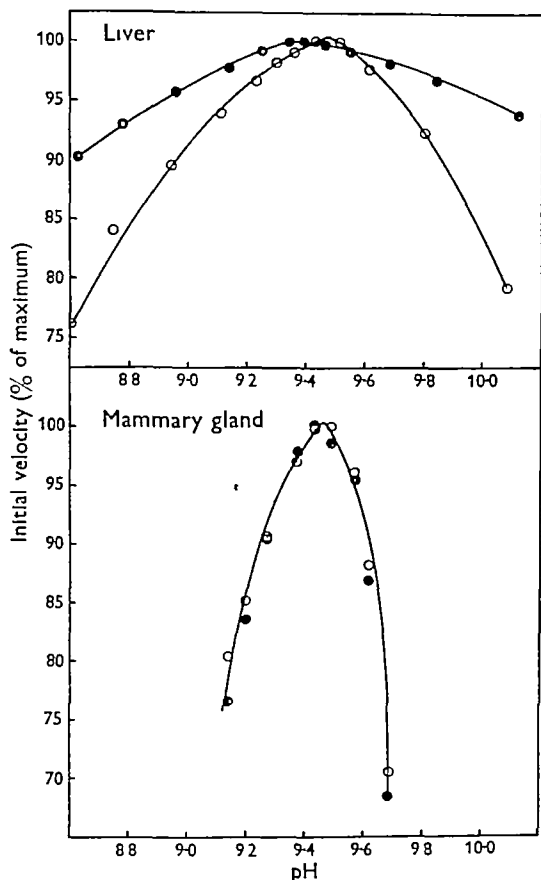


Fig. 7 Activity pH curves for arginase in homogenates of rat liver and mammary gland, with (black dots) and without (circles) added Mn^{++} . All experiments at 37° and in presence of 0.071 M substrate. Reaction time 10 min. The ordinates represent initial reaction velocities expressed as percentages of the initial velocity at the optimum pH.

is increased), whilst at pH 9.45 the velocity in presence of 0.071 M substrate is considerably less than the maximal. Thus it would be expected that, even if there is no shift in the optimal pH, the pH-activity curves at the lower substrate concentration would show appreciably less curvature than those at the higher substrate concentration. This is the case for the liver homogenates, but rather surprisingly the reverse holds for mammary gland homogenates (cf. Figs. 6 and 7).

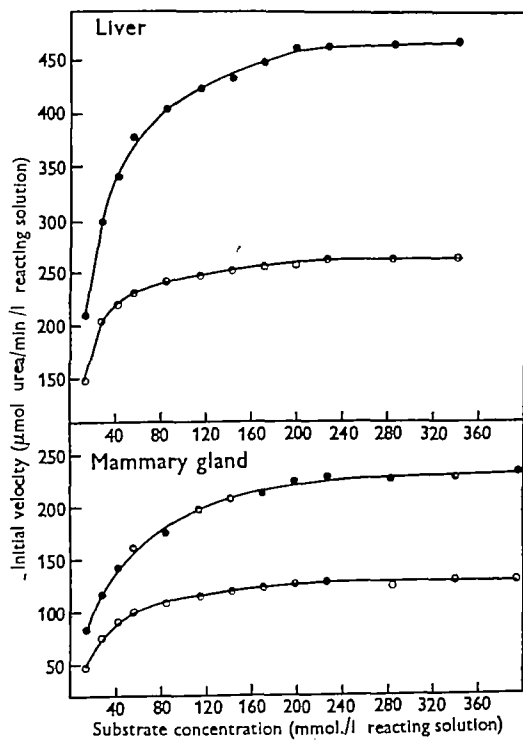


Fig. 8 Relation between substrate concentration and enzyme activity for arginase in homogenates of liver and mammary gland, with (black dots) and without (circles) added Mn^{++} . All experiments at 37° and pH 9.45. Reaction time 10 min.

effects on the final urea colour of widely different arginine concentrations. Each solution, before the colour was measured, was therefore brought to a standard arginine concentration by suitable dilution with water or an appropriate arginine solution. There

was of course still some possible error since the amounts of urea, L arginine and L ornithine in many of these final solutions were no longer equivalent, as the galvanometer calibration curves require them to be. However, this residual error may be assumed to be too small to justify the amount of labour necessary to eliminate it.

The curves in Fig. 8 resemble that given by Van Slyke & Archibald (1946) in that there is no sign of inhibition at high values of substrate concentration (S), but they differ from it in reaching a virtual maximum or saturation value in the neighbourhood of $S=0.22M$, while the curve of Van Slyke & Archibald continues, after the initial rapid increase, to rise slowly up to $S=0.4M$. The highest maximal velocity was attained by activated liver homogenate (Fig. 8), and corresponds to an enzyme concentration of approximately 460 units/l. It may therefore be concluded that, in the presence of $0.22M$ substrate, enzyme concentrations at least up to this value are fully saturated in the initial stages of the reaction. We were thus led to specify a final substrate concentration of $0.227M$ for our routine method of arginase activity determination, as being sufficient to give a maximum velocity with concentrations of enzyme likely to be encountered under our conditions, while corresponding to a conveniently prepared substrate solution (8% w/v as monohydrochloride).

A very different type of activity S relationship was revealed in the experiments at pH 9.1 (Fig. 9). Inhibition was now observed with high substrate concentrations so that the curves show definite optima at a substrate concentration ($0.071M$) which is the same for all. It is evident that, for arginase activity determinations on the acid side of the optimal pH, considerably lower substrate concentrations should be used than for methods employing the optimal pH.

The change with pH in the type of activity-substrate concentration relationship exhibited by arginase in tissue homogenates is of interest, but no explanation will be attempted here. Damodaran & Narayanan (1940), studying liver arginase, also found some indication of inhibition with increasing substrate concentration. Their optimal substrate concentration was given as $0.067M$, a value not very different from ours at pH 9.1, but a puzzling feature is that their results were obtained at pH 9.4, which is not far from our optimal pH at which no inhibition at high substrate concentration occurs. No such inhibition is apparent in the results of Greenberg & Mohamed (1945), but their studies were not extended into a range of sufficiently high substrate concentration for inhibition to occur.

Enzyme inhibition by excessive substrate concentrations is a fairly common phenomenon (see Haldane, 1930, Lineweaver & Burk, 1934). Folley &

Kay (1935) observed it with unfractionated preparations of mammary gland and kidney alkaline phosphatase, and they found that their results could be fitted passably well by Haldane's (1930) equation derived on the basis of the following postulated reactions: $E + S \rightleftharpoons ES$ (active), $ES + S \rightleftharpoons ES_2$ (inactive). Analysis of our data for the Mn^{++} activated arginase in homogenates of mammary gland and

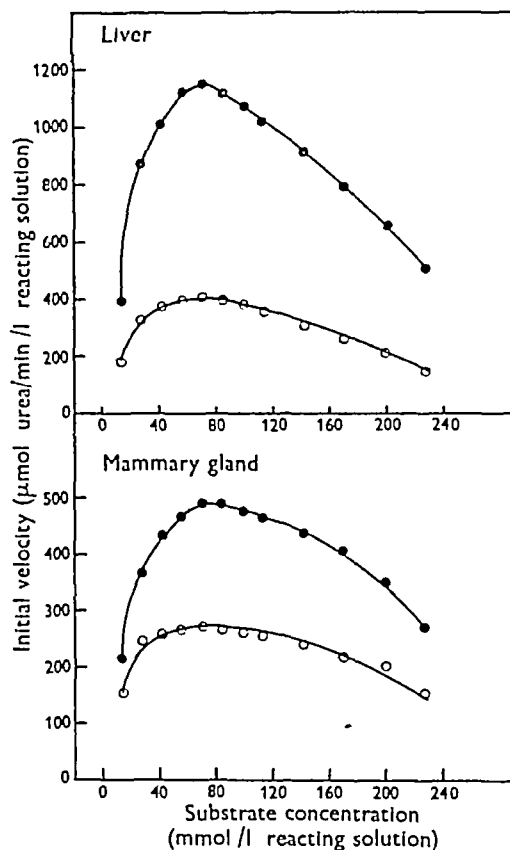


Fig. 9. Relation between substrate concentration and enzyme activity for arginase in homogenates of liver and mammary gland, with (black dots) and without (circles) added Mn^{++} . All experiments at 37° and pH 9.1. Reaction time 10 min.

liver by the methods of Case III of Lineweaver & Burk (1934) gave good agreement (see Fig. 10), over practically the whole range of substrate concentrations studied, with the postulated reactions $E + S \rightleftharpoons ES$ (active), $ES + 3S \rightleftharpoons ES_4$ (inactive). The values for K_s (or K_m) are given in Table 1, together with those for non-activated homogenates at pH 9.1. Whatever may be the actual mechanism of the substrate inhibition observed with arginase in our experiments, and the above analysis does no more than indicate a possible mechanism consistent with the results, it seems that, at pH values acid to the optimum but not at the optimum itself, arginase

Table 1 Values of K_m for various arginase preparations

Enzyme preparation	Determined at		K_m	References
	pH	Temp (°)		
Rat-liver homogenate + Mn^{++}	9.45	37	0.0200	Present investigation
Rat-liver homogenate	9.45	37	0.0100	
Rat-liver homogenate + Mn^{++}	9.1	37	0.0161	
Rat-liver homogenate	9.1	37	0.0177	
Rat-mammary gland homogenate + Mn^{++}	9.45	37	0.0200	"
Rat-mammary gland homogenate	9.45	37	0.0257	
Rat-mammary gland homogenate + Mn^{++}	9.1	37	0.0218	
Rat-mammary-gland homogenate	9.1	37	0.0061	
Dog liver extract + Mn^{++}	9.5	25	0.0460	Van Slyke & Archibald (1946)*
Purified liver arginase	9.45	40	0.0150	
Purified liver arginase	9.1	40	0.0130	Greenberg & Mohamed (1945)†
Purified ox liver arginase	9.4	?	0.0118	
Rat-liver slices	7.3	37	0.0025	Damodaran & Narayanan (1940)
Glycerol extract of rat liver	7.3	37	0.00275	
				Bach, Crook & Williamson (1944)

* Calculated by the method of Lineweaver & Burk (1934) from values read from published curve

† Values read from an apparently conjectural published curve showing relation between K_m and pH

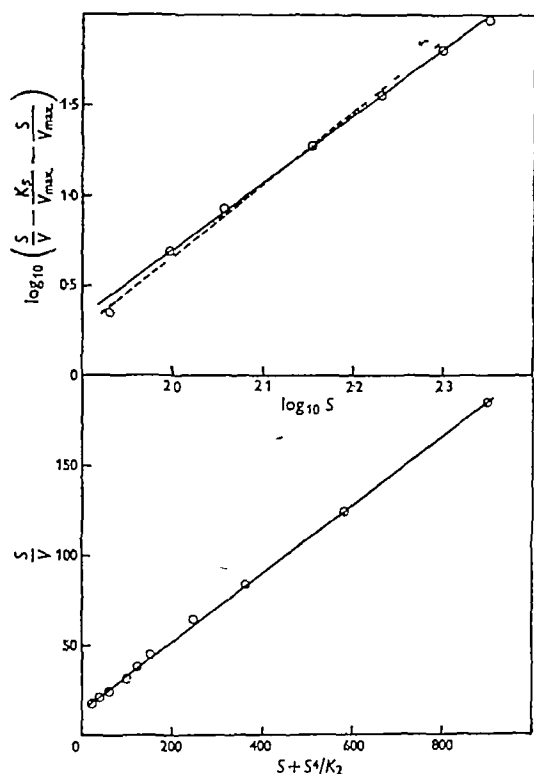


Fig 10 Analysis of possible mechanism of inhibition by excessive substrate concentrations of Mn^{++} activated arginase in rat-liver homogenate by the method of Case III of Lineweaver & Burk (1934). On the assumption that inhibition is due to the reactions $E + S \rightarrow ES$ (active), $ES + (n-1) S \rightarrow ES_n$ (inactive), the value of n is given by the slope of the straight line drawn through the experimental points in the upper graph. The dotted line is a line of slope = 4. Agreement with theory demands a linear relationship between the functions plotted in the lower graph.

may exist, at least partly, in a form which is able to combine with the substrate in a manner not conducive to hydrolysis.

Michaelis constants (pH 9.45) for activated and non-activated homogenates of liver and mammary gland were evaluated graphically by the method of Case I of Lineweaver & Burk (1934). Plots of S/V against S gave satisfactory linear relationships over the whole range studied (Fig. 11) in agreement with the Michaelis-Menten theory, and confirming the apparent absence at pH 9.45 of inhibition by excessive substrate concentrations. The values for K_m , which, however, were far from constant, are given in Table 1, together with values given by, or calculated from, the results of previous investigators for comparison. The values ascribed to Greenberg & Mohamed (1945) we read from what is evidently a conjectural curve showing K_m as a function of pH, that ascribed to Van Slyke & Archibald (1946) was evaluated by the Lineweaver-Burk method from values we took from their substrate concentration-activity curve. The values quoted from Bach, Crook & Williamson (1944) were determined on rat liver slices and on a glycerol extract of liver, but their curves are hardly complete enough to justify their use for a simple, direct graphical reading of K_m .

Activation with divalent metal ions

Relative activating effects of Mn^{++} and Co^{++} In our studies of the activating effects of Co^{++} (cf. Hellerman & Perkins, 1935; Hunter & Downs, 1944; Mohamed & Greenberg, 1945) relatively small activation effects were observed, rather surprisingly, when homogenates were incubated at 37°, and at a pH and with Co^{++} concentrations found by other workers (e.g. Mohamed & Greenberg, 1945) to suffice for maximal activation.

Experiments in which the degree of activation was measured after various periods of incubation at 37° with Co^{++} showed that, whilst the activity of both liver and mammary gland homogenates containing no added Co^{++} progressively fell after preparation, even at room temperature, the inactivation was retarded, if not completely prevented, by the presence of Co^{++} . In some experiments the addition of Co^{++} raised the activity slightly above its original value, in others the activity of Co^{++} treated homogenates showed a progressive fall, but slower and of

factor would, however, not be so important in the 30 min hydrolysis used for liver homogenates

In contrast to the lack of effect with Co^{++} , another activator, Mn^{++} (Klein & Ziesse, 1935), was found to exert a striking activating effect on homogenates of liver and mammary gland alike, much of which is so rapid (Fig 12) as to appear practically instantaneous by the methods used

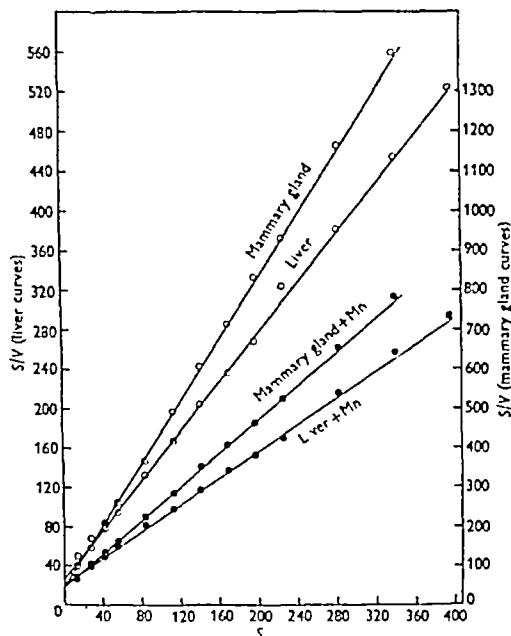


Fig 11 Linear relationship at pH 9.45 between S/V and S for the arginase of rat-liver and mammary gland homogenates, S =substrate concentration, V =initial reaction velocity

lesser degree than with untreated homogenates. In one exceptional experiment, addition of Co^{++} produced an unequivocal, though not very striking, increase in activity. A typical experiment is illustrated in Fig 12. Clearly, under our conditions, Co^{++} does little more than protect the enzyme against progressive inactivation. We thus have a reasonable explanation of the apparent activating effect of Co^{++} on homogenates of mammary gland (e.g. Fig 1) in contrast to the almost complete lack of effect on liver homogenates, observed in unpublished preliminary experiments with our previous method of determination of arginase activity (Folley & Greenbaum, 1947a). Considerable inactivation of the type just discussed would occur during the 20 hr hydrolysis at 37° used for mammary gland homogenates, and if Co^{++} prevented or retarded this process there would apparently be appreciable activation, this

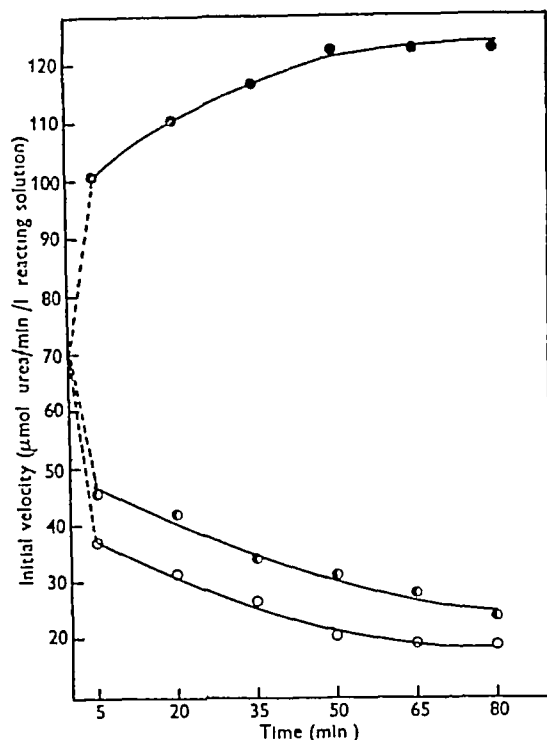


Fig 12 Effect on the activity of the arginase in a rat-liver homogenate, after dilution 1:200 with saline, of incubation at 37° for various periods (a) in the presence of 2 mg Mn^{++} /ml (black dots), (b) 2 mg Co^{++} /ml (half dots) and (c) in the absence of added divalent metal ions (circles). All activity determinations were carried out at 37° and pH 9.45, substrate concentration 0.227M, reaction time 10 min

In view of the marked and quite unexpected difference thus revealed between the activating effects of Mn^{++} and Co^{++} on the arginase in tissue homogenates, and in order to eliminate the possibility that this might not hold under other conditions, it seemed desirable to investigate the activating effects of Mn^{++} and Co^{++} over a wider range of conditions. Accordingly, factorial experiments (each set up in duplicate) were devised to enable the effect of incubating homogenates for different times with various concentrations of each cation to be studied. In each of two such experiments liver and mammary tissue from a lactating rat were homogenized, and portions of each homogenate at once suitably diluted, each time to the same degree, with saline containing appropriate amounts of MnSO_4 or CoCl_2 . The dilutions were forthwith incubated at 37°, and samples from each taken at appropriate times for enzyme determi-

nation, in one experiment at pH 9.45 and in the other at pH 9.1. It is not possible to show the results graphically, since the set expressing the effect of a given cation on a given homogenate can only be graphically represented by a three dimensional surface. The results of the experiment in which the enzyme activities were determined at pH 9.45 are, therefore, given in Tables 2 and 3. They define the conditions for maximal activation of the enzyme at homogenate pH and 37° in homogenates of both tissues. All the experiments on arginase activation described in this paper were carried out at homogenate pH, i.e. pH 7. Mohamed & Greenberg (1945) found that the rate of activation of their purified arginase by Mn^{++} increased with rising pH, we

prefer, however, to specify conditions for maximal activation at homogenate pH because of the greater stability of the enzyme protein at neutral reactions.

In confirmation of the previous results in Fig. 12, Tables 2 and 3 show beyond doubt that Mn^{++} is a much more effective activator of the arginase in our tissue homogenates than Co^{++} , the effect of which is negligible by comparison. These results confirm the previous finding that much of the effect of Mn^{++} is so rapid as to appear instantaneous with the methods used.

Table 2 *Effect of concentration of Mn^{++} or Co^{++} and of time of incubation at 37° with Mn^{++} or Co^{++} on the arginase activity of a rat liver homogenate*

(The values given are initial velocities (μmol urea/min./l. reacting solution) determined at 37° and pH 9.45, substrate concentration 0.227M, reaction time 10 min. Values marked * correspond to full activation.)

Time in presence of Mn^{++} at 37° (min.)	Final concentration of added Mn^{++} (mg/ml)						
	0	0.5	1.0	1.5	2.0	2.5	3.0
	Initial velocities (μmol urea/min./l.)						
0	36.5	79.6	83.7	88.9	94.7	100.5	104.2
15	31.4	90.5	93.2	97.9	103.4	110.0	111.2
30	26.8	96.1	102.0	108.1	111.2	116.3	117.8
45	20.0	102.2	107.8	114.2	117.8	123.3*	124.8*
60	18.9	108.1	111.4	115.8	121.4	124.8*	123.3*
75	18.5	111.2	114.6	123.3*	123.3*	123.3*	124.8*

Time in presence of Co^{++} at 37° (min.)	Final concentration of added Co^{++} (mg/ml)						
	0	0.5	1.0	1.5	2.0	2.5	3.0
	Initial velocities (μmol urea/min./l.)						
0	36.5	41.1	41.8	43.1	43.9	45.1	45.9
15	31.4	34.6	35.2	40.3	41.1	41.8	41.8
30	26.8	29.8	30.4	32.2	32.8	33.5	34.1
45	20.0	24.8	25.9	29.8	30.3	30.9	30.9
60	18.9	21.1	22.0	27.0	27.6	27.6	27.0
75	18.5	19.4	20.0	23.1	22.6	23.1	23.1

Table 3 *Effect of concentration of Mn^{++} or Co^{++} and of time of incubation at 37° with Mn^{++} or Co^{++} on the arginase activity of a rat mammary gland homogenate*

(The values given are initial velocities (μmol urea/min./l. reacting solution) determined at 37° and pH 9.45, substrate concentration 0.227M, reaction time 10 min. Values marked * correspond to full activation.)

Time in presence of Mn^{++} at 37° (min.)	Final concentration of added Mn^{++} (mg/ml)						
	0	0.5	1.0	1.5	2.0	2.5	3.0
	Initial velocities (μmol urea/min./l.)						
0	60.7	136.8	152.7	157.4	164.1	190.8	188.4
15	55.8	144.2	161.0	163.5	181.2	205.5	208.5
30	54.1	153.0	165.1	167.8	192.9	210.0	216.5
45	50.8	158.8	170.6	172.4	206.1	215.0	218.3*
60	51.1	164.0	174.0	177.8	215.4	218.2*	217.5*
75	49.6	169.4	175.8	182.4	219.3*	218.8*	216.5*

Time in presence of Co^{++} at 37° (min.)	Final concentration of added Co^{++} (mg/ml)						
	0	0.5	1.0	1.5	2.0	2.5	3.0
	Initial velocities (μmol urea/min./l.)						
0	60.7	65.1	66.1	68.0	68.0	68.3	68.0
15	55.8	63.3	64.6	67.0	66.8	66.7	67.0
30	54.1	61.7	63.3	65.1	66.1	66.3	66.1
45	50.8	62.4	64.6	64.8	65.9	67.1	67.2
60	51.1	62.4	65.2	66.1	67.1	67.4	67.2
75	49.6	63.0	65.6	67.1	67.1	67.1	67.6

These findings perhaps indicate that Mn is the actual metal component of the enzyme system in its 'native' state. It seems possible that the 'native' enzyme protein is almost specific in its requirements as regards the metal component, while on purification it becomes increasingly less specific, so that an active arginase can be constituted by addition to the partially purified protein of other divalent metal ions such as Co^{++} , Ni^{++} and Fe^{++} . Thoni, Roche & Roger (1947) have found that alkaline phosphatase, of which Mg^{++} was once regarded as an almost specific activator (e.g. Folloy & Kay, 1936), can be reactivated after purification and dialysis by a number of divalent metal ions. In any event, our findings indicate that if homogenates are to be used for studying changes in the fully activated, or what for the present may be termed the 'potential', arginase activity of tissues, it is necessary to use Mn^{++} for the activation process rather than Co^{++} which has been favoured by many investigators of tissue arginase changes (e.g. Kochakian, 1944). The significance of studies of changes in tissue-arginase activity with Co^{++} added as activator would seem to be somewhat doubtful.

The progressive inactivation of the enzyme in tissue homogenates at pH c 7 observed at 37° in these experiments, and which also proceeds at an appreciable rate at room temperature, is probably due to the effects of dilution (see also Van Slyke & Archibald, 1946). Dilution of the tissue enzyme systems is an inevitable accompaniment of homogenization, irrespective of any subsequent dilution necessary (e.g. with liver homogenates) to make the enzyme concentration suitable for activity measurements. Such dilution may be assumed to cause gradual dissociation of the metal protein complex, apparently forming the enzyme system (see Hellerman & Stock, 1938). This concept seems to be supported by our finding that this inactivation is reversible, in that not only can the original activity be restored by addition of Mn^{++} to an homogenate which has undergone considerable inactivation due to keeping either at room temperature or at 37°, but also the activity can thus be raised to the higher level characteristic of full activation.

Kinetics of dissociation inactivation and of reactivation by Mn^{++} The dilution of homogenates during preparation makes it necessary to decide what relationship exists between arginase activities of homogenates, and (a) the actual concentration of the enzyme protein in the tissues, and (b) the proportion of it which is combined with Mn^{++} thus constituting the active enzyme system (degree of activation). These are clearly questions of considerable importance.

In an attempt to throw light on this problem, the kinetics of dilution inactivation of the arginase in tissue homogenates, and its reactivation by Mn^{++}

were studied by a more refined technique made possible by the increased sensitivity of our new method of arginase activity determination.

Experiments were carried out in which rat liver was homogenized (5 min) in three portions. One homogenate was, immediately after preparation, diluted 1/8 with normal saline for activity determinations, a second was similarly diluted with saline containing sufficient MnSO_4 to give a final concentration of 2 mg Mn^{++}/ml . In the third case, the liver tissue was homogenized with saline containing sufficient MnSO_4 to give the same final concentration of Mn^{++} , and then diluted with the Mn^{++} saline containing 2 mg Mn^{++}/ml . All three homogenates were kept at room temperature during the experiment. As soon as possible after dilution, samples of each were taken for arginase determination using 1 min hydrolysis at pH 9.45, and other samples were similarly taken for enzyme determination at first at intervals of 0.5 min and later of 1 min.

Three such experiments were carried out with similar results save in one respect discussed below. One experiment is illustrated in Fig. 13 in which, since each enzyme activity determination occupied 1 min during which activation or inactivation was proceeding, the first point of each curve was plotted at time 0.5 min from the instant of dilution instead of at time 0, and the subsequent points plotted accordingly. The same plotting procedure was adopted for Fig. 12.

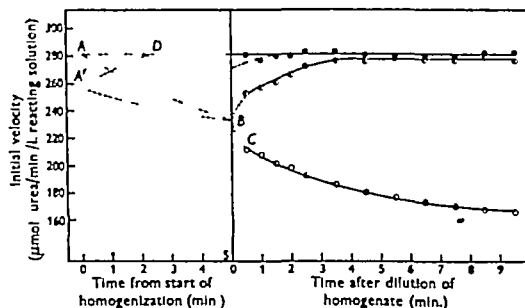


Fig. 13 Changes with time in the activity of the arginase present in homogenates of a rat liver. One homogenate was diluted with normal saline immediately after preparation (circles), another was similarly diluted with saline containing Mn^{++} (half dots). In the third case Mn^{++} was present during homogenization and Mn^{++} saline used for the dilution (black dots). After dilution the three preparations were kept at room temperature and samples taken at intervals for activity determination. Enzyme activities were determined at 37° and pH 9.45, substrate concentration 0.227M, reaction time 1 min. For explanation of dotted portion of curves, see text.

The curves for the two homogenates, in which no added Mn^{++} was present during homogenization, have been extrapolated back to intersect at zero time, i.e. the instant at which the dilution was performed. In making this conjectural extrapolation, it is assumed that the activities of the two homogenates changed at approximately the same rate, so that the curves are made to intersect at a point about midway

between their initial values. The point of intersection (*B*) represents the activity of the homogenate just prior to dilution. This, however, is probably somewhat less than the activity of the enzyme as it existed in the tissue, since some dissociation of the metal enzyme must have occurred during homogenization. It is evident that dilution of a freshly prepared homogenate causes considerable loss of activity even at room temperature, in the experiment illustrated in Fig 13 there was an approximately 15% drop over the first 5 min in the activity of the homogenate diluted with normal saline.

The activity of the preparation homogenized with Mn^{++} remained constant at a value which was approximately equal to the 'ceiling' value attained by the homogenate activated with Mn^{++} saline after preparation. Furthermore, the activity of the homogenate containing no Mn^{++} could be raised to the same level when Mn^{++} was added at the end of the experiment, thus confirming our previous finding that dilution inactivation is reversible.

These results seem to be susceptible of two interpretations. On the one hand it may be assumed that the enzyme was fully activated in the intact tissue, and that the activity of the preparation homogenized with Mn^{++} represents, as nearly as can be determined by present methods, the true tissue arginase activity. The fall in activity which occurs during homogenization in the absence of added Mn^{++} can then be reasonably represented by some such curve as *AB* in Fig 13. On the other hand it is equally possible that in the tissue the enzyme was not completely activated, and that in the preparation homogenized with Mn^{++} activation of the enzyme up to the full 'ceiling' value occurred during this process. In this case the 'native' activity may be represented by some point *A'* intermediate between *A* and *B* (Fig 13), and the course of activation during the homogenization in the presence of Mn^{++} by *A'D*. At present there is no obvious way of deciding between these alternatives, either of which may hold for the particular liver in question. The results of the other two experiments seemed to indicate that the arginase in the two livers concerned could not have been fully activated in the 'native' state, since in each case the Mn^{++} -homogenized preparation showed some rise in activity at the beginning of the series of determinations (depicted by the part-dotted line in Fig 13), which doubtless represented the concluding stages of an activation process initiated during homogenization. It is hardly possible to speculate in greater detail about the course of events during homogenization since, in the present state of knowledge, these are bound to be obscure, but it seems safe to conclude from these experiments that the degree of activation of the arginase system *in vivo* cannot be determined by present methods. More work will be necessary before

it will be possible to evaluate the prospects of determining the true arginase activity of a given tissue.

In the meantime, determinations of the 'potential' tissue arginase, i.e. of the level of enzyme protein in the tissues, a measure of which is given by the fully activated arginase activity of an homogenate (Mn^{++} not Co^{++} must be used), would seem to be of greater biological significance than determinations of arginase carried out in absence of Mn^{++} , though such values (e.g. those of Folley & Greenbaum, 1947*a, b*), provided they are made under strictly standard conditions as regards dilution and are performed as soon as possible after homogenization of the tissues, are almost certainly capable of leading to valid conclusions regarding relatively large changes in tissue arginase.

Reliance on determinations of the fully activated arginase, though this represents the best that can be done at present, is, however, not entirely satisfactory, since we are faced with the difficulty of deciding whether observed changes in tissue arginase activity are due to changes in the concentration of the active Mn^{++} protein or merely to alterations in the proportion of an unchanged amount of protein which is combined with Mn^{++} . At present there seems to be no satisfactory way of deciding between these alternatives.

SUMMARY

- 1 The arginase present in homogenates of rat liver and mammary gland has been studied by means of a sensitive reaction-velocity method involving colorimetric determination of urea.

- 2 The reaction velocity at the pH optimum and in presence of excess substrate (0.227*M*) is sensibly linear over the first 10 min, so that, provided not too much substrate is hydrolyzed, amounts of urea formed in periods up to 10 min are proportional to initial velocities, and can be converted into suitable arginase units without use of an enzyme calibration curve. Generally similar results were obtained at pH 9.1 in presence of 0.071*M* substrate.

- 3 The optimal pH (9.45) in presence of 0.227*M* substrate was the same for homogenates of both tissues, and was unaffected by full activation with Mn^{++} , the optimal pH was hardly affected by decreasing the substrate concentration to 0.071*M*.

- 4 At the optimal pH there was no evidence of enzyme inhibition by excessive substrate concentrations, the results agreeing well with the Michaelis-Menten theory, but at pH 9.1 such inhibition occurred and progressively increased as the substrate concentration was increased above the optimum (0.071*M*).

- 5 In the absence of added Mn^{++} , the arginase activity of liver and mammary-gland homogenates progressively falls even at room temperature and pH 7. The rate of inactivation is greater the more

dilute the homogenate, and it is doubtless due to gradual dissociation of the metal protein complex forming the enzyme. The inactivation can be reversed by addition of Mn^{++} (full activation taking an appreciable time even at 37°), or prevented if Mn^{++} is present from the outset. Co^{++} does not reactivate partially inactivated homogenates very much, and if present from the outset does little more than prevent or retard dilution inactivation. Mn^{++} and not Co^{++} is the metal component of the arginase system in the tissues, the enzyme system being reversibly dissociable.

6 Studies of the kinetics of dissociation inactivation and reactivation by Mn^{++} indicate that the enzyme may not always exist in the fully activated state *in vivo*. There is no known method by which the degree of activation of tissue arginase can be determined, and most biological significance attaches to the concentration of enzyme protein

(i.e. potential arginase) in the tissues, a measure of which is given by the activity of a homogenate fully activated by Mn^{++} .

7 The enzymes in liver and mammary gland homogenates are so similar in properties that they can be considered identical. In some respects the enzyme in tissue homogenates probably behaves more like the 'native' enzyme than does that in partially purified preparations. Homogenates are thus preferable to extracts for tissue arginase studies.

8 A routine method for the determination of tissue arginase is described. It involves homogenization of the tissue, and the determination of the arginase activity of the homogenate, fully activated by Mn^{++} , with only 5 min., and sometimes only 1 min., hydrolysis.

We are indebted to Mr S. C. Watson and Miss P. Hasker for technical assistance.

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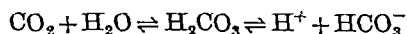
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Carbonic Anhydrase as a Tool in Studying the Mechanism of Reactions Involving H_2CO_3 , CO_2 or HCO_3^-

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In the study of enzymic processes where CO_2 or HCO_3^- or H_2CO_3 are reactants, either as end products or as starting material, it is often of interest to know the order in which the three forms of carbonic acid of the reversible system

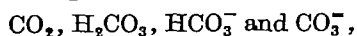


arise or react. For example, when urea is acted upon by urease in a buffer solution the end products are NH_4^+ , CO_2 , and HCO_3^- and the question presents itself whether CO_2 , or HCO_3^- , or H_2CO_3 is the primary form of carbonic acid. Similar problems arise in studying the mechanism of decarboxylation of α - or β ketonic acids and of the bacterial fission of formic acid into H_2 and CO_2 .

As Roughton and his colleagues (Meldrum & Roughton, 1933, Ferguson & Roughton, 1934, Roughton, 1935) have already pointed out, carbonic anhydrase can be used as a tool in studying this kind of problem. The present paper is an attempt to discuss the scope of this tool, to elaborate the experimental conditions under which it can be used and to define the conclusions which can be drawn from various types of experiments. Applications of the method to the study of urease and yeast carboxylase are given.

Theoretical considerations

General In this paper the term carbonic acid is used to mean the sum of the true carbonic acid (H_2CO_3) and the forms arising therefrom by electrolytic and molecular dissociation. Under physiological conditions, i.e. in the region of neutrality, carbonic acid is present in four forms



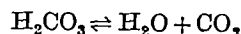
with CO_2 and HCO_3^- predominating. The composition of the equilibrium mixture in a closed system, with a gas phase and a liquid phase, is governed by the volumes of the gas and liquid phases, the dissociation constants of carbonic acid, pH, and the absorption coefficient of CO_2 .

The effect of carbonic anhydrase on the course of a reaction in which carbonic acid takes part depends on the nature of the primary product (whether CO_2 or H_2CO_3) and on the factors controlling the secondary reactions leading to the formation of the

equilibrium mixture. Among the latter, the pH and the nature and concentration of the buffers are outstanding in importance.

The use of carbonic anhydrase is limited by the following two requirements.

(1) The rate of the reaction in which carbonic acid is formed or used must be rapid in comparison with the rates of the uncatalyzed reaction



Otherwise no observable effect will be caused by addition of the enzyme.

(2) The reaction must take place under conditions where carbonic anhydrase is active, substances inactivating carbonic anhydrase must be absent.

In the following discussions specific cases showing the effects of carbonic anhydrase are analyzed in detail and illustrated by manometric experiments. The treatment refers to conditions where a relatively small amount of substrate is added to an enzyme sufficiently powerful to decompose the substrate completely in a matter of minutes.

Effects of carbonic anhydrase if CO_2 is the primary form of carbonic acid If CO_2 is the primary product and the pH is in the region of 5.0 carbonic anhydrase should not have an appreciable effect because at that pH CO_2 does not form bicarbonate to an appreciable extent.

If, in contrast, the pH is higher, say in the region of 7.0 or higher, some CO_2 will enter into secondary reactions yielding mainly HCO_3^- . The change of CO_2 pressure in a closed system will then depend on the relative rates of (1) the primary reaction yielding CO_2 , (2) the secondary reaction converting CO_2 into HCO_3^- , and addition of carbonic anhydrase should diminish the rate of CO_2 evolution in the early stages, but the precise effect of carbonic anhydrase will vary according to the relative rates of (1) and (2). Thus if (1) is much more rapid than (2), a rapid evolution of CO_2 in excess of the equilibrium will be followed by a slow absorption of the excess (Table 1 and Fig. 1), as the rate of (1) approaches that of (2), the amount of excess CO_2 diminishes (Table 3, Figs. 3 and 4). In both cases the addition of carbonic anhydrase will accelerate

the rate of (2) and therefore lower the CO_2 pressure in the early stages of the reaction, so that the system is in equilibrium throughout the reaction, but the effect of this enzyme is of course less marked in the second case. If the rate of (1) is equal to the rate of (2), CO_2 will not be evolved in excess of the equilibrium, and in this case carbonic anhydrase will have no demonstrable effect.

Effects of carbonic anhydrase if HCO_3^- or H_2CO_3 is the primary form of carbonic acid As the reactions $\text{H}_2\text{CO}_3 \rightleftharpoons \text{H}^+ + \text{HCO}_3^-$ are extremely rapid in comparison with the reaction $\text{H}_2\text{CO}_3 \rightleftharpoons \text{H}_2\text{O} + \text{CO}_2$ the effects of carbonic anhydrase are expected to be the same if either H_2CO_3 or HCO_3^- is the primary product of the reaction. In both cases carbonic anhydrase will accelerate the evolution of carbon dioxide if the pH is at or near the neutral point. An example is the standard procedure of Meldrum & Roughton (1933) of mixing a bicarbonate solution with phosphate buffer in the presence and absence of enzyme.

As the pH rises, the formation of CO_2 from bicarbonate decreases in magnitude until it becomes negligible above pH 8.0. Effects of carbonic anhydrase are therefore not expected at that pH. On the acid side of the neutral point, say between pH 5.0 and 6.0, the rate of the uncatalyzed decomposition of H_2CO_3 is very rapid and the manometric measurement of the acceleration by carbonic anhydrase becomes difficult.

EXPERIMENTAL

General technique The measurements consisted of the manometric determination of the change in the pressure of CO_2 within a closed system. The experiments were carried out with Warburg manometers, which were more convenient for the present work than the boat method. Conical flasks of about 20 ml capacity were used, having a side arm from which the substrate was added, but no centre well as the latter hinders the rapid movement of the fluids. In each experiment two manometers with flasks differing in volume by less than 1% were used. Identical quantities of the enzyme (urease or carboxylase), buffer, substrate and crude carbonic anhydrase were added to both flasks and an inhibitor of the latter enzyme (thiophen 2 sulphonamide) to one of the flasks. The two flasks therefore differed only by the presence of a small amount (usually 0.02 mg/ml.) of inhibitor. The possible alternative of adding carbonic anhydrase to one flask only may cause complications as impurities in the enzyme may have buffering capacities which affect the equilibrium. It is essential that the two flasks

should be equal in volume as the ratio $\frac{\text{CO}_2}{\text{total carbonic acid}}$ in the equilibrium mixture depends on v_g and v_f . The changes in CO_2 pressure therefore are not directly comparable if vessels of different sizes are used.

The quantity of substrate added was relatively small, equivalent to a maximum yield of 1000–2000 μl carbonic acid. Under these conditions the end point was reached within a few minutes and the pressure changes did not exceed the scope of the manometer scales.

An important control in this type of experiment is the measurement of the final value of the CO_2 output. The rates in two parallel manometric vessels are comparable only if the pH's and the concentrations of the buffers are identical. The final CO_2 value is determined by the pH and the buffer concentration and its measurement is therefore a check on the identity of conditions in the two flasks.

The manometers were first shaken slowly until equilibrium was established. The contents of the side arms of the two flasks were then mixed simultaneously by two people and the rate of shaking was increased to about 150 periods/min, with an amplitude of 7 cm at the level of the bottom of the flasks. Two small glass beads (2 mm diam) were placed in the solution to increase the effectiveness of shaking.

In experiments in which a substrate was added from the side arm to an enzyme the pH of substrate and enzyme solutions was carefully adjusted to the same value, as checked by colorimetric and electrometric measurements, and the initial amounts of CO_2 and HCO_3^- in both solutions were kept as low as possible. If the solutions contain HCO_3^- differences in pH cause pressure changes on mixing which may vitiate the results of the enzymic process.

The temperature of the bath was low, usually 15° or below. Owing to the relatively high temperature coefficient of the uncatalyzed reaction $\text{H}_2\text{CO}_3 \rightleftharpoons \text{H}_2\text{O} + \text{CO}_2$ (see Roughton, 1943) the effects of carbonic anhydrase are likely to be greater the lower the temperature.

Urease. Jack beans obtained from the Imperial College of Tropical Agriculture, Trinidad, were broken up in a coffee grinder and the husks were separated from the remainder by sieving. The bean meal (6 g) was shaken for 10 min with water or buffer solution (50 ml) and centrifuged. At 15°, 2 ml of the supernatant decomposed 2.5 mg urea completely in about 40 sec.

Carboxylase. Freshly pressed brewer's yeast was spread in thin layers and allowed to dry in the air. This required less than 24 hr. The dried material was stored in a cool, dry place. The carboxylase preparation was made on the day on which it was used by incubating a mixture of 20 g dried yeast and 60 ml water for 2 hr at 30° and centrifuging; the supernatant was used as carboxylase. At 15° and pH 5.5, 3 ml solution, on addition of 782 μl pyruvate, liberated 230 μl CO_2 during the 1st min and 316 μl during the 2nd min. The rate of pyruvate decomposition was of the same order at pH 7.0.

Carbonic anhydrase. Most experiments were made with the chloroform-ethanol preparation of Roughton & Booth (1946), or blood serving as the starting material. In a few cases laked ox blood was used. In the following protocols 'carbonic anhydrase' refers to the undiluted chloroform-ethanol extract, unless stated otherwise. To obtain maximum effects it was necessary to add about 0.1 ml of this to 2 or 3 ml of the reaction mixture.

Inhibitors. Thiophen 2 sulphonamide, kindly supplied by the American Cyanamide Company, was used as a standard inhibitor, 0.1 ml of a 0.05% aqueous solution was added to the solution. According to Davenport (1945), the discoverer of the inhibitory action of this compound, thiophen 2 sulphonamide is about 6 times as inhibitory as is sulphanilamide at 0°. After most of the work reported in this paper was completed it was found that *p*-toluene sulphonamide is still more effective as an inhibitor than thiophen 2 sulphonamide, and this substance was used in

some experiments Controls indicated that these sulphonamides had no significant effect on the activity of urease or yeast carboxylase when carbonic anhydrase was absent

Addition of buffers As the magnitude of the secondary reactions of carbonic acid depends on the concentration of buffers, phosphate was added to the enzyme solutions in most experiments, the amounts added are stated later

Experiments with urease

Our first experiments with urease were carried out in 1934 and have already been referred to briefly (Roughton, 1935) The experimental conditions in

Table 1 *Effect of carbonic anhydrase on the course of CO₂ evolution from urea in the presence of urease*

(Each flask contained 2 ml jack bean extract (6 g ground jack beans shaken with 50 ml 0.05M phosphate buffer pH 7.4, centrifuged and supernatant used, pH 6.9) and the additions stated below in the main compartment, 0.25 ml 10% urea ($\equiv 930 \mu\text{l CO}_2$) in the side arm, 15°, v_0 18.0, K_{CO_2} 1.952)

Time after mixing (min.)	CO ₂ evolved (μl)	
	Flask 1	Flask 2
	(0.2 ml carbonic anhydrase, 0.1 ml 0.05% thiophen 2-sulphonamide)	(0.2 ml carbonic anhydrase 0.1 ml water)
0.5	552	129
1.0	566	137
1.5	510	138
2.0	470	138
2.5	427	138
3.0	388	138
4.0	326	138
5.0	275	138
6.0	230	138
8.0	185	138
10.0	164	138
13.0	148	138
18.0	138	138
26.0	138	138

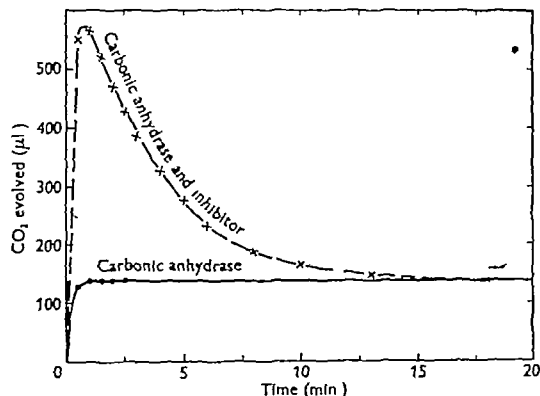


Fig 1 Effect of carbonic anhydrase on the evolution of CO₂ in the system urease urea (15°)

which effects of carbonic anhydrase can be demonstrated are given in Table 1 and the results are shown graphically in Fig 1. It will be seen that in the absence of carbonic anhydrase urea equivalent to 930 $\mu\text{l CO}_2$ yielded 566 $\mu\text{l CO}_2$ within 60 sec (61%

of the theoretical yield) Of this amount 428 μl were reabsorbed in the course of 18 min. In the presence of carbonic anhydrase there was no excess

Table 2 *CO₂ evolution from a mixture of methylamine carbamate and phosphate buffer with and without carbonic anhydrase*

(Both flasks contained in the main compartment 3 ml 0.1M phosphate buffer, pH 6.5, and the additions stated below, and in the side arm 0.3 ml 1.2N methylamine carbamate carbonate solution prepared by passing CO₂ through a solution of methylamine until it turned alizarine yellow to pink orange. The total CO₂ content of the solution (carbamate + carbonate) was 0.470M and the pH was 10.4 (see Faurholt, 1924), 15°)

Time after mixing (min)	CO ₂ evolved (μl)	
	Flask 1 Carbonic anhydrase (0.2 ml)	Flask 2 Water (0.2 ml)
0.33	159	721
0.67	232	960
1.0	270	1033
1.33	288	1040
1.67	301	1025
2.0	310	1000
3.0	321	908
4.0	324	825
6.0	324	682
10.0	324	508
14.0	324	409
20.0	324	355
30.0	324	331

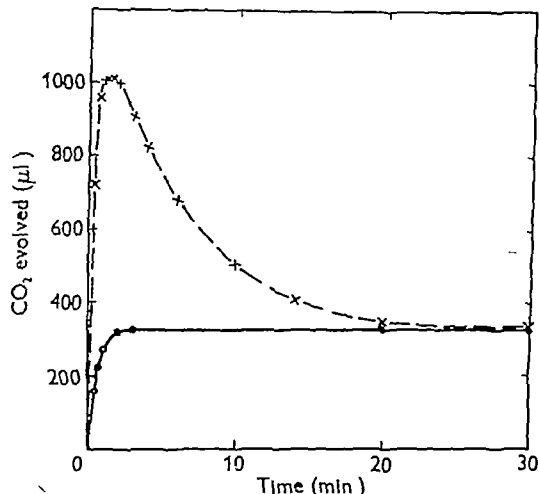


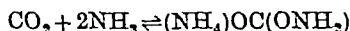
Fig 2 Effect of carbonic anhydrase on the evolution of CO₂ in a mixture of methylamine carbamate and phosphate buffer (15°). Carbonic anhydrase added, - - -, water added -x-x-

production of CO₂, and the final yields of CO₂ were the same with and without the enzyme

In further experiments the concentrations of the reactants were varied. When less carbonic anhydrase was used there was a small excess production of

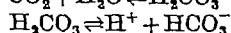
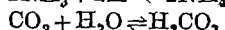
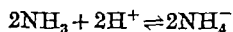
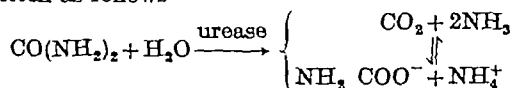
CO₂ even in the presence of the enzyme. When the urease solution was diluted 10 times with phosphate buffer pH 7.1 the course of the reaction was protracted and the excess CO₂ was smaller in amount but still marked. The effects of carbonic anhydrase were also demonstrable at 30°. On the other hand, as expected, no effects of carbonic anhydrase were observed at 15° at pH 6.0 (phosphate buffer) or 5.0 (acetate buffer).

The results make it certain that bicarbonate or H₂CO₃ are not primary products in the action of urease. They are in accordance with the view that primary reaction products are either ammonium carbamate or CO₂ + NH₃. When a solution containing a carbamate is added to an approximately neutral buffer, curves of the same general shape as those given by the urea-urease system are obtained, as Ferguson & Roughton (1934) have shown, and as is further illustrated by Table 2 and Fig. 2. The view that ammonium carbamate is an intermediate was first expressed by Fenton (1885) and by Armstrong & Horton (1912), and substantiated by Yamasaki (1920), Mack & Villars (1923), and especially by Sumner, Hand & Holloway (1931). The last named workers showed conclusively that ammonium carbamate is formed when urease is allowed to act in alkaline medium. It could not be detected in the presence of buffers. The authors rightly point out that the demonstration of the presence of ammonium carbamate does not necessarily prove that this substance is the first intermediate. The reversible reaction



is extremely rapid (Faurholt, 1924), and in the experiments where carbamate was detected it could have arisen either directly from urea or secondarily from CO₂ and NH₃. It is not possible at present to distinguish between these alternatives.

The action of urease and the subsequent series of reactions in the presence of buffers may therefore be written as follows



Experiments with yeast carboxylase

To 20 ml yeast extract were added, immediately before use, Na₂HPO₄ · 2H₂O (178 mg) and, drop by drop, CO₂ free N NaOH (1.7 ml). The resulting turbid mixture turned phenol red pink and showed electrometrically a pH of 7.0. A 0.2M pyruvate solution of the same pH and phosphate concentration was prepared from a N stock solution of pyruvic acid, Na₂HPO₄ · 2H₂O and CO free N NaOH.

The details of two experiments showing the effects of carbonic anhydrase are given in Table 3 and Figs 3 and 4. In both experiments carbonic anhydrase retarded the evolution of CO₂ in the manner expected for a reaction yielding CO₂ as the primary product. At 7.5° the excess of CO₂ evolved was greater than at 15° and the effect of carbonic anhydrase correspondingly more marked. A greater effect is expected at a lower temperature if the temperature coefficient of carboxylase (which is about 2.1) is smaller than that of the uncatalyzed hydration of CO₂.

As regards the rate of CO₂ evolution from its substrate the carboxylase was less potent than the urease, and in addition, the rate of reaction dropped more rapidly with decreasing substrate concentration in the case of carboxylase. These two factors account for the differences in the shapes of the curves obtained with the two enzymes, although in both cases CO₂ is the primary product.

In similar experiments at pH 6.0 and 5.0 no effects of carbonic anhydrase were seen.

It will be noted that there is a short lag period before the full rate of CO₂ evolution is reached. It is very unlikely that any significant part of this lag is due to diffusion. Attempts to abolish the lag were unsuccessful. Addition to the main compartment of small amounts of glucose or pyruvate which were decomposed during the equilibration period had no effect.

The experiments leave no doubt that CO₂, and not H₂CO₃ or HCO₃⁻, is the primary product of decarboxylation. This result is in contrast to that of Conway & MacDonnell (1945), who claimed in a preliminary publication to have observed an acceleration of the CO₂ output by carbonic anhydrase, when yeast carboxylase acts upon pyruvate. Prof. Conway has kindly supplied the full experimental details in the procedure employed by Conway & MacDonnell, and it has therefore been possible to repeat their experiments. Conway & MacDonnell worked at pH 5.5 and 37° and added a large excess of pyruvate. Under such conditions we did not find any difference between the rates of CO₂ evolution in the presence and absence of carbonic anhydrase and the results of Conway & MacDonnell thus could not be confirmed. At pH 5.5 and 37° the rate of the uncatalyzed dehydration of H₂CO₃ is very fast, the time for the half reaction being about 0.8 sec, no additional manometric effect is therefore to be expected on addition of carbonic anhydrase, and the effects recorded by Conway & MacDonnell cannot have been due to carbonic anhydrase, their origin remains unexplained.

It should be noted that the graphs published by Conway & MacDonnell are not directly comparable with those given in this paper, as Conway & MacDonnell plotted the rate of CO₂ evolution (not the total CO₂ output) against time.

Table 3 *Effect of carbonic anhydrase on the course of CO₂ evolution from pyruvate in the presence of yeast carboxylase*

(For details of carboxylase and pyruvate solution, see text. Each flask contained 3 ml. carboxylase and 0.1 ml. carbonic anhydrase in the main compartment and 0.4 ml. 0.2 M pyruvate in the side arm. Flasks (1) further contained 0.1 ml. 0.05% thiophen 2 sulphonamide solution. K_{CO_2} in Exp. 1, 2.13, in Exp. 2, 2.24.)

Time after mixing (min)	CO ₂ evolved (μl.)			
	Exp. 1 (15°)		Exp. 2 (7.5°)	
	Flask 1	Flask 2	Flask 1	Flask 2
0.5	11	8	11	10
1.0	45	23	23	18
1.5	92	45	45	26
2.0	140	77	72	42
2.5	200	113	103	56
3.0	251	155	137	80
4.0	356	234	200	122
5.0	412	302	256	164
6.0	469	356	306	205
7.0	511	405	347	241
8.0	522	443	383	274
10.0	542	490	441	335
12.0	554	516	490	389
14.0	—	—	526	421
16.0	—	—	540	458
17.0	—	—	545	466
19.0	—	—	545	485
21.0	—	—	535	490
25.0	585	582	515	499
30.0	—	—	500	502
40.0	591	588	500	502

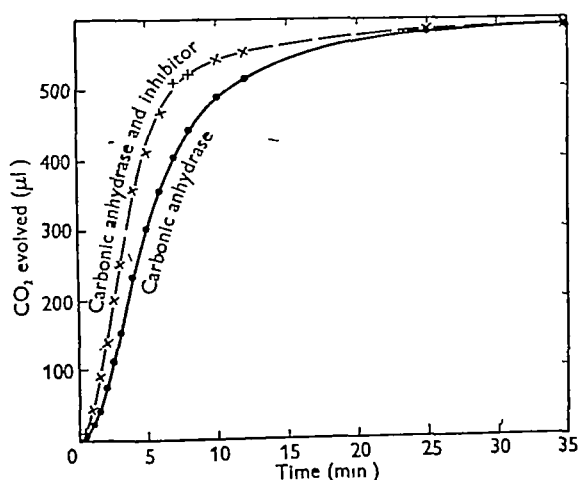


Fig. 3 *Effect of carbonic anhydrase on the evolution of CO₂ in the system yeast carboxylase pyruvate (15°)*

The use of Warburg manometers for the estimation of carbonic anhydrase activity

The reaction between bicarbonate and hydrogen ions and the effects of carbonic anhydrase thereon have already been thoroughly investigated by pre-

vious workers (see Roughton, 1943), but the use of the Warburg manometer for the study of this reaction has not been described in detail. The following experimental conditions, which are similar to those used in various forms of the 'boat' method of Meldrum & Roughton (1933), were found to be suitable for the given dimensions of the Warburg flasks.

Solutions. Phosphate buffer (0.1 M) prepared by mixing 300 ml. 0.1 M Na₂HPO₄ with 200 ml. 0.1 M KH₂PO₄, NaHCO₃ 0.1 M and 0.05 M.

Manometric arrangement. For experiments at 0°, 2 ml. phosphate buffer, and additions such as carbonic anhydrase and inhibitors in a total volume of 0.2 ml., were measured into the main compartment and 1 ml. 0.1 M NaHCO₃ into the side arm. For experiments at temperatures between 15° and 40°, 1 ml. buffer was used instead of 2 ml., and 1 ml. 0.05 M NaHCO₃ was placed in the side arm. An experiment at 15° is shown in Table 4. It will be seen that the initial rate was accelerated more than sixfold by the enzyme. At 38° the initial acceleration was about twofold.

At low enzyme concentration the acceleration caused by carbonic anhydrase is proportional to the enzyme concentration as shown in Table 5 which gives details of an experiment carried out at 0°. The uncatalyzed reaction, as well as the increase caused by relatively small amounts of the enzyme, proceeded approximately linearly until about one third, i.e. about 80 mm., of the final pressure change had been reached. Later the rate of reaction fell off. In

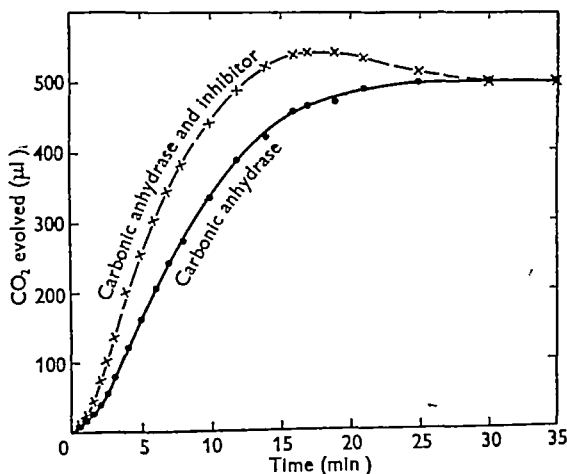


Fig. 4 *Effect of carbonic anhydrase on the evolution of CO₂ in the system yeast carboxylase pyruvate (7.5°)*

quantitative experiments the initial third section of the curve was used in calculating the activity of carbonic anhydrase. A reading in the region between 60 and 80 mm. was chosen and from it the rate of reaction for a standard period, usually 30 or 60 sec., was calculated. The application of this

procedure to the data recorded in Table 5 gives the following figures

	CO ₂ (μl) evolved in 30 sec	Increase due to carbonic anhydrase (μl CO ₂)
No-enzyme added	20	—
Enzyme diluted 1/320	49	29
Enzyme diluted 1/160	80	60
Enzyme diluted 1/80	138	118

The increased CO₂ output is thus proportional to the amounts of carbonic anhydrase added. The procedure is, therefore, suitable for quantitative measurements on the concentration of the enzyme in tissues and on the action of inhibitors. It must be emphasized that the rigid precautions in cleanliness—acid washing, rinsing with doubly distilled water, prevention of contamination by gas and metallic impurities—which Roughton & Booth (1946) found necessary, are essential for obtaining reproducible results. Even when these precautions are taken, single results cannot be relied upon, duplicate or triplicate experiments are essential.

Table 4 *Effect of carbonic anhydrase on the reaction between NaHCO₃ and phosphate buffer, measured in Warburg manometers*

(The carbonic anhydrase preparation was the chloroform ethanol extract from ox red cells, diluted 20 fold, the main compartment contained 1 ml buffer (see text) and the side arm 1 ml 0.05M NaHCO₃, 15°, *K*_{CO₂} 2.32)

Time after mixing (min)	CO ₂ evolved (μl)	
	Flask 1 Carbonic anhydrase (0.2 ml)	Flask 2 Water (0.2 ml)
0.33	196	30
0.67	280	63
1.0	332	98
1.33	354	128
1.67	372	154
2.0	382	179
3.0	399	244
4.0	402	296
9.0	405	392
15.0	405	406

In previous work the reciprocal of the time taken for a given evolution of CO₂ has been used as a measure of the enzyme activity, but if the rate of CO₂ evolution is linear, as is here the case in the

Table 5 *Effect of varying quantities of carbonic anhydrase on the rate of CO₂ evolution from a mixture of phosphate buffer and bicarbonate*

(0°, volume of manometer flasks, 24 ml, the carbonic anhydrase preparation was obtained from ox blood cells by treatment with chloroform and ethanol (Roughton & Booth, 1946), for further details, see text, the data are averages of duplicate determinations)

Time after mixing (min)	Pressure (mm) observed manometrically				
	With 0.1 ml enzyme				
	Without enzyme	Diluted 1/320	Diluted 1/160	Diluted 1/80	Undiluted
0.5	10	25	42	69	204
1.0	20	50	80	130	248
1.5	31	73	110	170	259
2.0	42	94	133	195	264
2.5	52	113	157	212	267
3.0	62	128	169	224	267
4.0	79	158	194	241	—
5.0	96	180	210	251	—
7.0	124	212	232	259	—
12.0	168	255	247	262	—
17.0	208	263	263	264	267

early stages, it is equivalent, and simpler, to use the amount of CO₂ evolved in 30 or 60 sec as a measure of activity.

SUMMARY

1 When a powerful urease preparation is allowed to act upon a limited quantity of urea in a neutral buffer at 15°, CO₂ is rapidly evolved and later gradually reabsorbed. If carbonic anhydrase is added, no excess of CO₂ is evolved. These results support the conception that CO₂, and not HCO₃⁻ or CO₃²⁻, is a primary intermediate in the action of urease on urea.

2 Carbonic anhydrase has a similar effect in the system yeast carboxylase pyruvate, under certain conditions, which are described. The results indicate that CO₂, and not HCO₃⁻ or H₂CO₃, is the primary product in the enzymic decarboxylation of pyruvate.

3 The effects which are to be expected when carbonic anhydrase is added to systems in which CO₂ or bicarbonate are reactants are discussed from a general point of view.

4 The use of Warburg manometers for the quantitative determination of carbonic anhydrase activity is described.

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Electrophoretic Studies on Human Serum Albumin

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Prolonged electrophoresis of human serum has demonstrated the non-homogeneity of the albumin in phosphate buffer at pH 8 and ionic strength 0.1, the migration velocities of the boundaries of the two main components, as apparent from the patterns, differing by about 2% or less (Blax, Tiselius & Svensson, 1941, Hoch & Morris, 1945). Under the conditions of these experiments, i.e. similar mobilities and comparatively low ionic strength, the conventional methods of analysis of the patterns cannot be applied, since here the separation of the boundary peaks and the relative areas in the gradient curve are not even approximate measures of the difference in mobility and of the relative proportions of the two components in the original mixture. A detailed analysis of boundary anomalies of this kind was given by Svensson (1946). The calculation of the true proportions and relative mobilities by means of the theory available at present (Svensson, 1943, Dole, 1945, Svensson, 1946) can yield only approximate information on account of the necessity for assumptions concerning changes in mobility of the ions across the boundary. A simplified method of treating the data has been suggested for use under certain conditions (Hoch, 1948).

In the present paper, the ascending patterns of albumin obtained after prolonged electrophoresis of normal and pathological sera are studied by a procedure similar to that used previously.

THEORETICAL

Definition of symbols

V_1, V_2 = migration velocities of proteins 1 and 2 at infinite dilution in the buffer used as supernatant, V_{a1}, V_{a2} = actual migration velocities of proteins 1 and 2 below the ascending boundaries, C_1' = concentration of protein 1 in the region between the ascending boundaries of 1 and 2, C_1 = concentration of protein 1 in the region below the ascending boundary of protein 2, C_t' = total concentration of protein in the region below the ascending boundary of protein 2, C_1', C_2' and C_t' are expressed in g/100 ml, K = coefficient as defined in the text, U_1 = mobility of a protein at infinitely low protein concentration in the buffer used as supernatant, U = mobility of this protein in the actual solution below the protein boundary, a = constant as defined in text, I = current/unit area of cross section, S = separation of two boundaries/unit time.

Notation for Svensson's (1946) equation (21) c_{i1} = ionic concentrations in electrochemical equiv/ml above colloid boundary, with the signs of the charges, u_{i1} = ionic mobilities in $\text{cm}^2 \text{V}^{-1} \text{sec}^{-1}$ above colloid boundary, with the signs of the charges, u_2/u_1 = mobility ratio across the colloid

boundary, C = concentration of a leading ion in equiv/ml with the sign of the charge, U = mobility of colloid ion above the boundary, defined by equation (22) of Svensson (1946), κ_2/κ_1 = ratio of conductivities of solutions above and below the colloid boundary.

The relation between migration velocity and concentration of serum albumin

The conditions obtaining in the region between the ascending boundary of a leading protein and the following ion depend only on the properties and concentration of the leading protein ions in this layer, and on the buffer solution used as the supernatant fluid. Thus was the same in all experiments. Assuming (a) that in this region the relative change in migration velocity with protein concentration (expressed in g/100 ml.) is the same for the two main components of serum albumin at pH 8, and (b) that the relative rate of change of the migration velocity of one component, when at infinitely low concentration, with increasing concentration of the second component, is equal to the relative rate of change of the migration velocity of the second component with increasing concentration of this component, then

$$V_{a2} = V_2 (1 + K C_1') \quad \text{and} \quad V_{a1} = V_1 (1 + K C_1'), \quad (1)$$

where the subscripts 1 and 2 refer to the faster and slower albumin components respectively, K was considered to vary little with the protein concentration, when this is low (Hoch, 1948). In the derivation of the expression for K on the basis of Svensson's (1946) theory, the mobility ratio for the protein, U/U_1 , was taken to be equal to that of the buffer ions across the boundary.

An expression for K can also be derived, if the ratio U/U_1 for the protein ions is taken to differ from that of the buffer ions by amounts proportional to the protein concentration (expressed in equiv/ml), i.e.

$$\frac{U_1}{U} (1 + aC) = \frac{u_1}{u_2} = \frac{U}{U} \quad (2)$$

By combining (2) with Svensson's equation (21) (1946)

$$\left(\frac{\kappa_2}{\kappa_1} - \frac{u_2}{u_1} \right) \Sigma \frac{c_{i1} u_{i1}}{u_{i2} - U} + C = 0,$$

we have

$$\left(\frac{\kappa_2}{\kappa_1} - \frac{u_2}{u_1} \right) \frac{u_1}{u_2} \Sigma \frac{c_{i1} u_{i1}}{u_{i1} - U_1 (1 + aC)} + C = 0$$

In the following, $\Sigma \frac{c_{i1} u_{i1}}{u_{i1} - U_1 (1 + aC)}$ will be written Σ

$$\left. \begin{aligned} \frac{\kappa_2}{\kappa_1} \frac{u_1}{u_2} &= \frac{\kappa_2}{\kappa_1} \frac{U_1}{U} (1 + aC) = 1 - \frac{C}{\Sigma}, \\ \frac{\kappa_1}{\kappa_2} \frac{U}{U_1} &= (1 + aC) \frac{\Sigma}{\Sigma - C}, \\ \frac{\kappa_1}{\kappa_2} \frac{U}{U_1} - 1 &= \frac{aC\Sigma + C}{\Sigma - C} = \frac{1 + a\Sigma}{\Sigma - C} C \end{aligned} \right\} \quad (3)$$

K is defined as previously, or from (1),

$$K = \frac{1}{C_1'} \frac{V_{a1} - V_1}{V_1},$$

inserting

$$V_{a1} = \frac{UI}{\kappa_2} \quad \text{and} \quad V_1 = \frac{U_1 I}{\kappa_1},$$

$$K = \frac{1}{C_1'} \left(\frac{\kappa_1}{\kappa_2} \frac{U}{U_1} - 1 \right),$$

and from (3)

$$K = \frac{(1 + a\Sigma) Q_s \times 10^{-2}}{\Sigma - Q_s \times 10^{-2} \times C_1'}, \quad (4)$$

where the concentration of protein is expressed in g/100 ml and Q_s is the net charge/g protein. Solving (4) for Q_s we have

$$Q_s \times 10^{-2} = \frac{K\Sigma}{1 + a\Sigma + KC_1'}$$

If $a\Sigma$ is negative, the value of Q_s thus found is larger, if positive smaller, than that found from equation (4a) of the previous paper (Hoch, 1948)

$$K = \frac{Q_s \times 10^{-2}}{\Sigma \frac{c_{a1} u_{a1}}{u_{a1} - U} - Q_s \times 10^{-2} \times C_1'},$$

derived by using the assumption that the mobility ratios of all ions are equal, i.e. the coefficient of C_1' in the denominator in (4) is larger (or smaller), so that variations in C_1' have more (or less) effect on K . The variation in Σ with C_1' is here comparatively small, as the numerical calculation showed. No data are available to estimate a , but for the present purpose it may be sufficient to evaluate the variation in K with C_1' for an arbitrary limiting case, in which this variation is greatest. For example, using the experimental value of $K = 0.06$ (Hoch, 1948), and taking the value of Q_s as being 25% higher than that of Q_s given in the literature (for references see Hoch, 1948), with the help of equation (4) $a = +0.45 \times 10^4$ and the change in K is calculated to be +11%, when C_1' increases from 0 to 1 g albumin/100 ml.

In the present study the approximation is made that K is independent of the albumin concentration and the numerical value of 0.06 is used in all calculations. In consequence, all the values given below for the relative proportions and relative mobilities should be considered as approximations.

Calculation of the relative proportions and the relative difference in mobility of the two albumin components. The concentration of the faster albumin component ahead of the boundary of the slower component adjusts itself to such a value that the increase in concentration across this boundary is balanced by the decrease in migration velocity. The amount of the faster albumin ions/unit cross section which passes the boundary of the slower component must be the same whether calculated in terms of the migration velocity behind the slower boundary or that ahead of it. Using equation (1), this is expressed by

$$\begin{aligned} (V_1 - V_2)(1 + KC_1')C_1' \\ = V_1(1 + KC_1')C_1' - V_2(1 + KC_1')C_1' \\ = (V_1 - V_2)(1 + KC_1')C_1' - V_1K(C_1' - C_1')C_1' \end{aligned}$$

Hence
$$C_1' = C_1' - \frac{V_1K(C_1' - C_1')C_1'}{(V_1 - V_2)(1 + KC_1')} \quad (5)$$

The separation per unit time, S , of the two boundaries, after they have migrated V_{a1} and V_{a2} respectively, is $\frac{C_1'}{C_1'}(V_1 - V_2)(1 + KC_1')$ (Hoch, 1948) from which

$$V_1 - V_2 = S \frac{C_1'}{C_1'} \frac{1}{(1 + KC_1')} \quad (6)$$

Inserting (6) into (5)

$$\left. \begin{aligned} C_1' &= C_1' - \frac{V_1K(C_1' - C_1')C_1'}{S} \\ \text{and} \quad C_1' &= \frac{C_1'}{1 + \frac{V_1}{S}K(C_1' - C_1')} \end{aligned} \right\} \quad (7)$$

From equation (7) the proportion of the faster component in the total can be obtained. The relative difference in migration velocity at infinitely low albumin concentration (=relative difference in mobility) is best calculated from equation (6) after C_1'/C_1' has been found from equation (7).

EXPERIMENTAL

In most of the cases 4 ml of fresh serum were diluted with 8 ml phosphate buffer of pH 8 and ionic strength 0.1, and dialyzed against 2 l of this buffer for 1-4 days at 0-4°. The serum protein concentration was determined by micro Kjeldahl or by the specific gravity method of Linderstrom Lang (Hoch & Marrack, 1945). The 11 ml Tischius cell and the Philpot-Svensson optical system with a diagonal edge or wire (Svensson, 1939), or both, were used. The potential gradient was about 10 V cm⁻¹ and the bath temperature 0.1°. After 70-80 min the serum patterns were photographed, and then a steady flow of buffer into the electrode vessel of the anode was started and kept up so that the albumin boundary did not migrate out of the top compartment of the U tube. The patterns were evaluated by the method of Svedberg & Pedersen (1940) as used by Longworth (1946), in which the areas of the peaks of the gradient curve are limited by lines similar to Gauss curves. This procedure involves much uncertainty in the analysis of patterns from the serum albumin, the components of which have not been completely separated (Pl 10) and no more than a crude estimate of the apparent relative proportions of the albumin components was attempted. Moreover, the distance migrated per hour by the ascending albumin boundary was used in place of V_1 and this distance was not measured in every experiment, but was assumed to be 2.5 cm at 20 mA. in all experiments. An accurate estimate was not considered necessary in view of the uncertainty involved in the measurements of the distances between the boundary peaks. The albumin concentrations on the ascending side were 0.8-1.4 g/100 ml in all but four cases.

As an example for the calculation of the true proportions and relative mobilities, case 22 may be described. Total albumin concentration, $C_1' = 1.2$, apparent proportion of faster component = 60%, $C_1' = 0.72$, distance between boundary peaks = 0.43 cm = $S \times \text{time}$, total distance migrated = 55 cm = $V_1 \times \text{time}$, but from equation (7) the concentration of the faster component in the mixture,

$$\begin{aligned} C_1' &= \frac{0.72}{1 + 55/0.43 \times 0.06 \times 0.48} = 0.15 \quad \text{or} \quad 13\% \text{ of } 1.2, \\ \frac{C_1'}{C_1'} &= 4.8, \quad 1 + KC_1' = 1.072, \quad \frac{S}{V_1} = \frac{0.43}{55}, \end{aligned}$$

from equation (6)

$$\frac{V_1 - V_2}{V_1} = \frac{0.43 \times 4.8}{55 \times 1.072} = 0.035 \text{ or } 3.5\%$$

Only those patterns which showed two main peaks were analyzed in this way

RESULTS

The results are summarized in Table 1 and Pl 10. The proportion of the component represented by the faster main peak ranged from 0 to 42% of the total albumin. The albumin probably includes α_1 globulin, except in case 25 in which this had been removed. The mobilities of the two components differed by 2.5–6.5%. The findings in Table 1 were arranged into four groups in the order of increasing proportions of the faster component, irrespective of the clinical state of the subjects. Owing to the uncertainty in the estimations, little significance should be attached to the order within any one group.

The percentages of the faster component in the albumin from five healthy individuals were between 10 and 42. Cancer sera gave percentage values of the faster component of 0–5 in five, of 6–10 in two, of 11–20 in two, and of 21–30 in one out of ten cases. Five out of seven sera from patients suffering from non-malignant diseases fell into the range found with sera from clinically healthy subjects. The two low values were found in one case of cirrhosis of the liver and in one case of nephritis on two occasions.

Apart from the main components, a small component, migrating up to 12% faster than the albumins, was frequently observed. It was present in seven out of eight sera (including those which were electrolyzed undiluted) from clinically healthy subjects, in six out of eight sera from patients suffering from non-malignant diseases and in five out of ten cases of cancer.

The fastest boundary peak in cases 26–30 (Pl 10) was comparatively large and, except in case 26, its mobility differed little from that of the first main peak. On account of this large first component, the calculation of the relative proportions of the 'main' components was omitted in these cases, since equations (6) and (7) cannot be applied to more than two components. In four instances, portions of the same samples were tested before and after storage at 0–4° (Pl 10, bottom row). The keeping of serum produced very little change in cases 13 and 18, while a marked change in the pattern was observed in case 8. When serum was first diluted, dialyzed and then kept at pH 8, there was little change in case 8, but in case 13 the separation of the two components and the proportion of the faster component was increased. No change on keeping at pH 8 was seen in a sample of serum albumin that had been freed from α_1 -globulin by electrophoresis in veronal buffer (Longworth, Curtis & Pembroke, 1945) and sub-

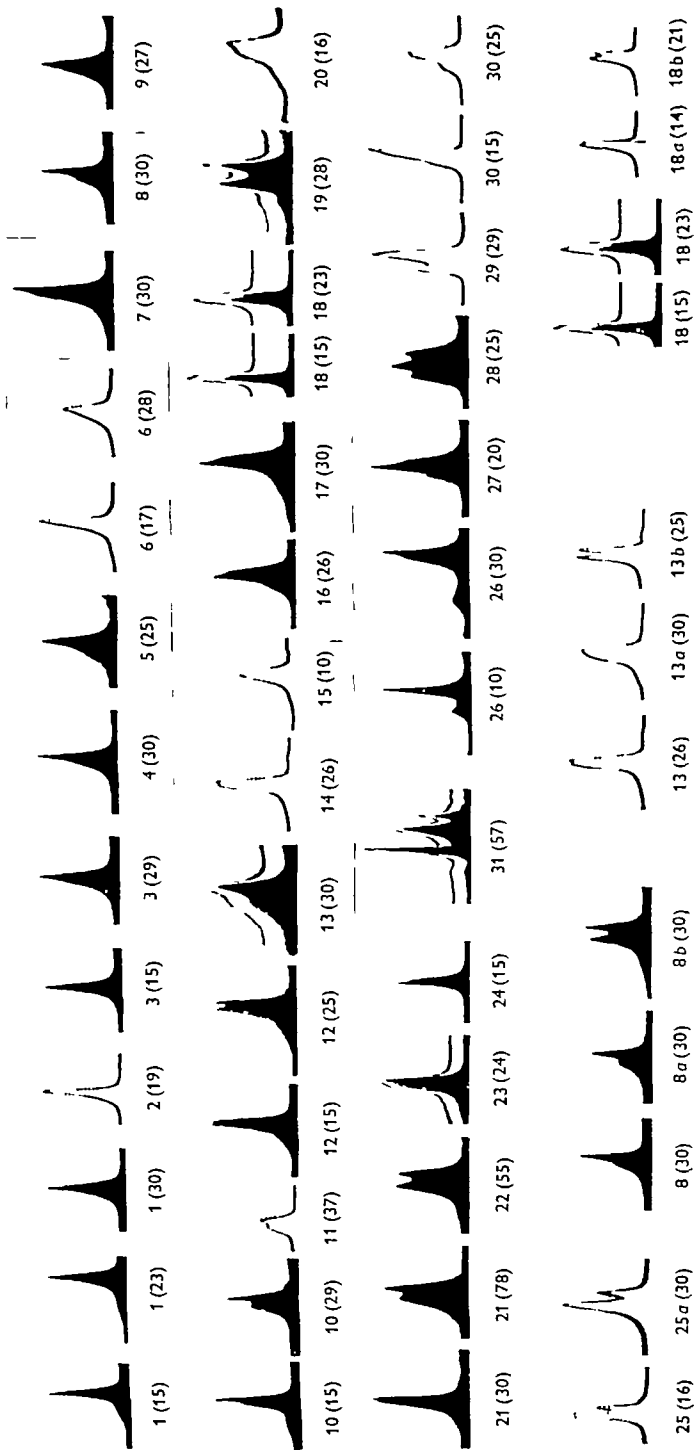
sequently dialyzed against phosphate. The changes that occurred on keeping were in all instances in the direction of a greater separation of the main peaks. The following sera had been kept or dialyzed at 0–4° for more than 6 days before electrophoresis (the number of days is given in parentheses): 2 (9), 6 (8), 7 (12), 11 (18), 19 (8), 20 (21), 29 (11).

The albumin pattern no. 31 of Pl 10 was obtained in an experiment with undiluted serum. A similar pattern was obtained with serum from the same subject taken 2 months later. In both cases there were three main and two small fast components. In the pattern shown here a slight disturbance can be seen in the region below the boundaries (i.e. to the right in the figure) which is probably due to convection. Undiluted sera from two other subjects gave albumin patterns with three and two main albumin peaks respectively. In addition one small fast and one small slow peak were present in either pattern. Although the small slow peaks behaved like genuine components, it is possible that they were due to convection, but it is unlikely that these convections arose from the heat generated by the current, since under identical conditions in many other experiments no small slow peaks were observed.

DISCUSSION

The assumption that two components are similar with regard to the change of mobility with protein or with salt concentration was used in the derivation of equations (6) and (7). The values for the relative proportions and the relative difference in mobility of two serum albumins calculated by means of these equations differ considerably from those apparent from the patterns. These calculated values are considered more reliable than those obtained by the conventional method (in which the relative areas are taken to represent the relative amounts in the mixture) even if the assumptions about the changes in mobility should prove to be not quite correct, provided the values for the K of the two components are similar. A formally similar treatment has been used by Johnston & Ogston (1946) to explain a boundary anomaly found in the ultracentrifugal sedimentation of mixtures.

In most of the patterns shown in Pl 10, particularly patterns 6, 13 or 16, the extent of the separation of the two components might appear insufficient for a quantitative analysis. Greater separation could have been obtained by continuing the experiments for a longer time, but for technical reasons this was not done. The uncertainty in the extrapolation of the curves between the peaks can be assessed by considering the limits within which both curves could be obtained reasonably symmetrical. In case 6 of Pl 10 these limits (ratio of the areas on both sides of the maximum ordinate not greater than about



Albumin patterns after prolonged electrophoresis of sera The patterns 1-25 are numbered corresponding to Table 1

Nos 1-20, 26-30, albumin patterns from diluted pathological sera, 21-24, albumin patterns from diluted normal sera, 26, pleurisy, 27, menstrual oedema, 28, carcinoma of breast, 29, nephritis 30, carcinoma of anus
No 31, albumin pattern from undiluted normal serum, 25, pattern of a normal serum albumin, free from α_1 globulin, 25a, dialyzed solution of 25 kept (at pH 8) for 15 days
No 8a, serum of 8 diluted and dialyzed 1 day (dialyzed solution kept (at pH 8) for 15 days), 8b, serum of 8 kept 18 days, then diluted and dialyzed 1 day
No 13a, serum of 13 kept 20 days, then diluted and dialyzed 2 days, 13b, serum of 13 diluted and dialyzed (at pH 8) for 27 days
No 18a, serum of 18 kept 18 days, then diluted and dialyzed 1 day
The number in parentheses gives the distance in cm migrated by the ascending albumin boundary The direction of migration is from the right to the left

Table 1 Analyses of two component patterns of serum albumin

No	Sex	Age	Diagnosis*	Additional fast component	Distance between boundaries A_1 and A_2 (cm) ($S \times \text{time}$)	Distance migrated since mobility ($\text{cm} \times \frac{V_1 \times}{\text{time}}$)	Relative difference in mobility (%)	Concn of albumin (g/100 ml) C_1'	Area of faster peak as % of total area of albumin peaks	Proportion of faster component as % of total albumin \dagger	Number of cases	
											Ca *	Healthy
1	♀	46	Ca of antrum	+	—	—	—	1.2	—	—	5	4 (including 2 pregnancies)
2	♂	18	Nephritis (13 in. 46)	—	Faster component distinguishable	—	—	0.8	—	—		
3	♂	18	Ca of soft palate	—	0.25	29	5.5	0.9	10	<2		
4	♂	70	Pregnancy, toxæmia	—	0.35 (± 0.15)	30	6	1.0	10	2		
5	♀	34	Pregnancy, toxæmia	—	0.45	25	6	0.8	10	3		
6	♀	63	Ca of antrum ventriculi, liver metastases	+	0.35 (± 0.15)	28	6.5	1.1	15 (†)	3	2	2
7	♂	35	Cirrhosis of liver	+	0.25	30	5.5	1.1	15	4		
8	♀	25	Pregnancy, chronic rheumatic carditis	±	0.4	30	6.5	1.2	20	4		
9	♀	60	Ca of bladder	±	0.3 (†)	27	—	0.8	—	Probably <5		
10	♀	67	Ca of rectum	—	—	—	—	—	—	—		
11	♀	49	Ca of cervix	+	0.35	29	6	1.2	30	6	2	1
12	♂	18	Same as no 2 (22 v 46)	±	0.3	37	5	1.5	50	8		
13	♂	70	Ca of penis	+	0.25	25	5	1.4	50	10		
14	♂	56	Cirrhosis of liver	+	0.6	30	6	0.7	30	10		
15	♂	—	Healthy	—	0.43	78	3	1.2	60	10		
16	♀	61	Ca of breast	—	0.18 (± 0.02)	26	3.5	1.3	60	11	2	2
17	♂	22	Mitral stenosis, heart failure	+	0.3	10	6	0.7	25	12		
18	♀	30	Granulosa cell tumour	+	0.2	26	3	1.3	70	17		
19	♀	35	Adenoma of thyroid	+	0.2	30	2.5	1.2	75 (†)	20		
20	♀	35	Healthy	+	0.43	55	3.5	1.2	60	13		
21	♂	—	Healthy, donor	+	0.25	24	4.5	1.2	50	11	1	1
22	♀	—	Ca of breast	Very small	0.2	23	2.5	1.4	75	22		
23	♀	—	Nephritis	+	{0.48 0.63}	{20 28}	4.5	0.8	50	25		
24	♂	19	Myelomatosis	+	0.45	16	5	0.8	60	27		
25	♂	38	Healthy, donor, electrophoretically purified albumin, free from α_1 globulin	Not looked for	0.13	19	2.5	1.2	75	21		
26	♂	62	—	—	0.3	16	3	1.0	75	42		

$$\dagger \left(\frac{SC_1'}{V_1 C_1' (1 + K C_1')} \right) + \left(\frac{C_1'}{1 + \frac{V_1}{S} K (C_1' - C_1)} \right)$$

* Ca = carcinoma.

2 or 2.5) were about 15 and 40 % of the total area. The value of 25 % was used in the calculation.

In the three cases which were tested, the separation into two albumin components could be reproduced after keeping either the serum or the dialyzed solution (at pH 8), but only in one case were the patterns of practically identical shape. The other patterns showed differences, all of which were in the direction of a greater separation of the peaks. It is therefore improbable that any sera which showed little or none of the faster component had altered in this respect on keeping or on dialysis.

The finding of a lower concentration of the faster main albumin component in a number of sera of patients with cancer warrants further chemical investigation of this component. Extensive work has been carried out with the purpose of establishing alterations in the protein composition of sera from persons suffering from malignant disease (for references see Stern & Willheim, 1943). Several changes have been found, but none of them was specific enough to be of definite diagnostic value. The present investigation might bear some relation to the findings of Kahn (1930). He found that the albumin fraction which is not precipitated by 37.15 % ammonium sulphate is diminished in sera of cancer patients. In these, as well as in the present experiments, sera from cases of pregnancy and liver and kidney diseases behaved similarly to those from cancer patients.

SUMMARY

1. The albumin patterns obtained after prolonged electrophoresis of diluted sera at pH 8 showed in general two main peaks, of which the faster covered

an area of from 0 to 75 % of the total area of the albumin peaks. These proportions of the areas, however, do not represent the proportions in the amounts of the albumin components in the original serum.

2. A method for the calculation of the relative proportions and the relative mobilities of two albumin components is presented. Applying this method to the analysis of albumin patterns it was found that the amount of the faster 'main' component ranged from 0 to 42 % of the total albumin. In five sera from clinically healthy individuals the percentage was 10–42, in seven out of the ten cancer sera examined, the percentage was only between 0 and 10. Similarly low values were found in two samples of sera from a patient with nephritis, in one case of cirrhosis of the liver and in two pregnancies.

3. In two cancer and three non cancer sera three main albumin peaks were found.

4. Electrophoresis of undiluted sera gave albumin patterns with three 'main' components in two out of three cases examined.

5. An additional small fast component was present in seven out of eight sera from clinically healthy subjects, in six out of eight sera from patients suffering from non malignant diseases and in five out of ten cases of cancer.

6. No feature of the albumin pattern specific only for cancer sera could be established.

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The Fate of Certain Organic Acids and Amides in the Rabbit

5 *o* AND *m* HYDROXYBENZOIC ACIDS AND AMIDES

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We have already reported (Bray, Ryman & Thorpe, 1947) upon the fate of *p* hydroxybenzoic acid and its amide in the rabbit. The present paper gives the corresponding results for the *o* and *m*-isomers. The changes which these compounds might undergo are theoretically the same as those for the *p*-isomer, i.e. the hydroxyl group may be conjugated with either glucuronic or sulphuric acids and the carboxyl group with glucuronic acid or glycine. In the case of the amides the extent of conjugation through the carboxyl group would depend upon the degree of hydrolysis of the carbamyl group. There is also the possibility of the introduction of a second hydroxyl group, comparable to the formation of protocatechuic acid from *p*-hydroxybenzoic acid or amide (Bray *et al.* 1947).

The extensive literature upon salicylic acid resulting from its clinical use in the treatment of rheumatic conditions was critically reviewed up to 1926 by Hanzlik (1926), who concluded that salicylic acid was excreted largely unconjugated, though some conjugation with glycine, to form salicyluric acid, might occur. The degree of this conjugation was variable in amount, depending to some extent on species, the evidence was most complete for man. There was no general agreement about glucuronide formation. It seemed probable that some destruction of salicylic acid occurred in the organism, and there was evidence that gentisic acid (2,5-dihydroxybenzoic acid) was formed by hydroxylation of salicylic acid. There has been general agreement among workers that salicylic acid is not conjugated with sulphate in man, dog or rabbit (e.g. Baumann & Herter (man and rabbit), 1877-8; Mosso (man and dog), 1889-90; Vogelius (man), 1880; Williams (rabbit), 1938), though Baumann & Herter claimed to have observed ethereal sulphate formation in the dog. The results of the comparatively few investigations described since 1926 have in general supported the conclusions in Hanzlik's (1926) review. Quick (1932*a, b*) found some evidence of glucuronic acid conjugation of salicylic acid in the dog, but very little conjugation with glycine was observed. He was, however, able to isolate salicyluric acid from human salicylate urine (Quick, 1933). Kapp & Coburn (1942) isolated salicylic acid (20%), salicyluric acid (55%) and gentisic acid (4-8%) from human salicylate urine, and in addition obtained a small amount of uraminosalicylic acid, apparently a complex of glycine, salicylic and gentisic acids, which was probably identical with a compound (uraminosalicylsäure) isolated by Baldoni (1908) from the urine of dogs dosed with salicylate. It is possible that these may

have been artifacts formed during isolation, but their formation and exact constitution have not been studied further. Kapp & Coburn (1942) obtained evidence of the conjugation of salicylic acid with glucuronic acid (25%), but no glucuronide could be isolated. Lutwak Mann (1943) showed that rats can also hydroxylate salicylic acid to form gentisic acid.

Very few investigations of the metabolism of salicylamide have been reported. Bondzynski (1897) concluded that it was completely absorbed in man. Baumann & Herter (1877-8) and Baas (1889-90) found that it caused an increase in ethereal sulphate in the dog, and Williams (1938) found that 29-40% was excreted in this form by the rabbit. On the basis of the salicyluric acid which he could isolate, Baas claimed that 2-12% was hydrolyzed in the dog.

m Hydroxybenzoic acid has been found to be conjugated with glycine and glucuronic acid in the dog (Quick, 1932*a, b*), with sulphate in man and dog (Baumann & Herter, 1877-8) and in the rabbit to the extent of 3-5% (Williams, 1938).

The only record of the metabolism of *m* hydroxybenzamide is Williams's (1938) observation that 32-44% was excreted as ethereal sulphate by the rabbit.

In this study we sought to establish the degree of hydrolysis of the carbamyl group in *o*- and *m*-hydroxybenzamides by quantitative estimation of the excretion of various metabolites, and comparison of the results with those obtained after administering the corresponding acids. We were able to isolate 2 and 3 carbamylphenylglucuronides as metabolites of *o*- and *m* hydroxybenzamides respectively, and to detect the formation of hydroxylation products from all four compounds, isolating gentisic acid from salicylate urine and gentisamide from salicylamide urine.

METHODS

Diet and dosing. The rabbits used were does of 2-3 kg. The diet of rabbit pellets already described (Bray *et al.* 1947) was used throughout this investigation. Salicylic acid and amide were appreciably toxic, and the maximum dose level used was 0.4 g/kg. The meta isomers were not toxic at dose levels up to 1.5 g/kg.

Methods of analysis. These were the same as those previously used (Bray *et al.* 1947), but in this investigation the ether soluble acid was not fractionated.

Preparation of *o* and *m* hydroxybenzamides. Salicylamide was prepared by refluxing methyl salicylate with excess ammonia (sp. gr. 0.88) until there were no longer

two layers. On cooling, the reaction mixture deposited long needles which were recrystallized from water. The final product melted at 139°. Yield 90% of theory. The final traces of methyl salicylate were removed by heating at 100° under reduced pressure. *m*-Hydroxybenzamide was prepared in the same way as *p*-hydroxybenzamide (Bray *et al* 1947). Yield 38% of the ester used. M.p. 170°.

Gentisic acid This reference compound was prepared by the method of Brunner (1907). M.p. 200–202°. Methyl gentisate, m.p. 87–88°, was prepared from it as described by Raistrick & Simonart (1933).

Gentisamide This was prepared by refluxing methyl gentisate (1 g) with ammonia sp. gr. 0.88 (10 ml.) for 2 hr. Concentration of the solution gave *gentisamide*, which on treatment with charcoal and recrystallization from water formed fine white needles, m.p. 217–218°. Yield 80% of theory. Found C, 54.5, H, 4.63, N, 9.07. $C_7H_7O_3N$ requires C, 54.9, H, 4.58, N, 9.15%. *Gentisamide* gave an intense blue colour with $FeCl_3$, reduced ammoniacal $AgNO_3$, but gave a yellow, instead of pink, colour with Lutwak Mann's (1943) test for gentisic acid.

RESULTS

Quantitative studies

Ether-soluble acid excretion For the rabbits used in these studies the average normal excretion of ether soluble acid (calculated as hippuric acid) ranged from 635 to 847 mg/day. Table 1 shows the percentage of doses of the four compounds under investigation, excreted as ether soluble acid, calculated in all cases as hydroxybenzoic acid. No attempt was made to fractionate the ether soluble acid, but since, as is described later, the amounts of glycine conjugates excreted appear to be negligible, the error introduced by assuming that the increase in acid excretion is entirely due to *o*- or *m*-hydroxybenzoic acids will be correspondingly small.

Excretion of reducing material The average normal daily excretion of reducing substances (calculated as glucuronic acid) by the rabbits used in this investigation ranged from 135 to 219 mg, and the average hydrolyzed reducing values from 176 to 378 mg. Table 2 summarizes the results obtained.

Excretion of ethereal sulphate The average normal daily excretion of ethereal sulphate ranged from 40 to 64 mg. Table 3 shows the percentage of doses of the compounds excreted conjugated with sulphate. The results for the acids agree with those obtained by Williams (1938), but those for the amides are much lower, viz. 13–16 and 10–19% as compared with 29–40 and 32–44% respectively. The diet in Williams's study consisted of bran, oats and cabbage, while in the present study pellets were given. This difference in diet is probably the reason for the divergence of the results obtained, since with *p*-hydroxybenzoic acid and amide, when the rabbits received the bran, oats and cabbage diet, the values

we obtained were in agreement with those of Williams (Bray *et al* 1947). Braunstein, Parschin & Chalsowa (1931) have investigated the effect of diet on the metabolism of phenol, and concluded that rabbits on an acid forming diet conjugate this compound to a greater extent than those on an alkali forming diet. This is particularly marked in the case

Table 1. *Excretion of ether-soluble acid (calculated as hydroxybenzoic acid) after doses of o- and m-hydroxybenzoic acids and amides**

Rabbit no	Approx dose (g/kg)	Percentage of acid excreted as ether soluble acid	Percentage of amide excreted as ether soluble acid
Ortho compounds			
117	0.25	76	2
135	0.25	84	0
145	0.25	80	0
147	0.25	98	0
117	0.5	93	0
135	0.5	88	0
145	0.5	79	0
147	0.5	81	0
Meta compounds			
119	0.25	78	—
130	0.25	76	—
140	0.25	57	—
143	0.25	69	—
119	0.5	94	—
130	0.5	64	9
140	0.5	87	19
143	0.5	92	14
119	1.0	87	—
119	1.0	101	11
130	1.0	107	10
140	1.0	97	19
143	1.0	95	4
143	1.0	—	6
119	1.5	97	0
130	1.5	99	3
140	1.5	97	8
143	1.5	96	3

* All values in Tables 1, 2 and 3 are calculated neglecting the formation of gentisic acid which is excreted conjugated with sulphuric acid (Likhatscheff, 1895–6) and probably also with glucuronic acid. The fact that gentisic acid itself is reducing is also ignored.

of ethereal sulphate formation. Injection of phenol (500 mg) into rabbits on the 'acid' diet caused an increase in ethereal sulphate excretion of 82 mg above the normal value, while with rabbits on the 'alkaline' diet, the corresponding increase was only 5 mg. The increase on a 'mixed' diet was 22 mg. Our observations are in accordance with these findings, inasmuch as the diet used by Williams and by us in our earlier investigation was an 'acid' diet and the pellet diet in this study 'alkaline'.

Table 2 *Percentage of doses of o- and m hydroxybenzoic acids and amides excreted as ester- and ether type glucuronides**

Rabbit no	Approx dose (g/kg)	Percentage of acid excreted as		Percentage of amide excreted as	
		Ester glucuronide	Ether glucuronide	Ester glucuronide	Ether glucuronide
		Ortho compounds			
117	0.25	8	5	8	71
135	0.25	7	1	7	83
145	0.25	0	0	3	69
147	0.25	0	12	7	82
117	0.5	14	11	5	64
135	0.5	12	15	4	58
145	0.5	10	14	7	76
147	0.5	15	16	1	71
		Meta compounds			
130	0.25	14	24	13	56
135	0.25	8	22	14	66
138	0.25	13	10	17	53
140	0.25	8	19	22	63
130	0.5	6	10	0	32
136	0.5	6	—	—	—
138	0.5	13	—	0	39
140	0.5	5	14	1	30
140	0.5	3	—	—	—
143	0.5	—	8	0	40
119	1.0	6	6	—	—
130	1.0	4	8	1	47
130	1.0	—	—	0	—
136	1.0	7	11	—	—
138	1.0	7	9	1	35
140	1.0	5	1	2	25
143	1.0	—	—	1	22
143	1.0	—	—	0	15
130	1.5	5	1	0	21
136	1.5	6	0	—	—
138	1.5	10	5	0	27
140	1.5	4	0	1	23
143	1.5	—	—	1	29

* See footnote to Table 1

*Qualitative studies**Isolation of metabolites of salicylic acid and salicylamide*

Gentisic acid (2,5-dihydroxybenzoic acid) Urine (24 hr) was collected from 40 rabbits which had received 40 g salicylic acid as its sodium salt, and hydrolyzed by boiling for 20 min with 0.2 vol conc HCl. It was then continuously extracted with ether for 48 hr, and the ethereal extract thoroughly washed with NaHCO₃ solution. The aqueous extract thus obtained was acidified with 5N-H₂SO₄ and extracted with ether. After removal of ether the ether soluble material was dissolved in water, and the resulting solution extracted with chloroform until all the salicylic acid was removed as indicated by the FeCl₃ colour given by successive extracts. The aqueous solution then gave an intense deep blue colour with FeCl₃ and reduced Benedict's solution and ammoniacal AgNO₃. On evaporation a yellow syrup remained which yielded fine white needles,

m p 198–201°. After recrystallization from ether-light petroleum the m p was 202–203°. Yield 1.5–2.0 g from 40 g salicylic acid. The identity of this product with gentisic acid (m p 200–202°) was established by the mixed m p with an authentic specimen (Found C, 54.4, H, 3.95. Calc for C₇H₆O₄ C, 54.6, H, 3.9%). The methyl ester (m p 87–89°) mixed with methyl gentisate (m p 87–88°) gave m p 87–89°.

When a similar procedure was applied to urine from rabbits which had received salicylamide, about 5 mg of gentisamide (m p 215–216°) were isolated. Its identity was confirmed by mixed m p determination, colour with FeCl₃, response to Lutwak-Mann's test and reduction of ammoniacal AgNO₃.

In a second experiment with salicylamide urine only a very small amount of crystalline material m p 170–180° was obtained. It gave a similar blue colour with FeCl₃ and contained only 1.54% N. There was not sufficient material for further investigation. The product may be a complex similar

to the 'uraminosalicylic acid' isolated by Baldoni (1908) and by Kapp & Coburn (1942) which contained 3.7–4% N. It is, however, perhaps more reasonable to suggest that the complex from salicylamide might be a mixture of gentisic acid and gentisamide.

Table 3 Percentages of doses of *o*- and *m*-hydroxybenzoic acids and amides excreted conjugated with ethereal sulphate*

Rabbit no	Approx dose (g/kg)	Percentage of acid excreted as ethereal sulphate	Percentage of amide excreted as ethereal sulphate
Ortho compounds			
145	0.25	0	11
147	0.25	0	14
117	0.5	0	12
135	0.5	0	19
Meta compounds			
130	0.25	6	21
140	0.25	2	16
136	0.5	3	—
138	0.5	1	—
140	0.5	—	16
143	0.5	—	20
119	1.0	2	—
130	1.0	3	—
138	1.0	—	10
140	1.0	1	—
143	1.0	—	9
143	1.0	—	14
130	1.5	3	11
140	1.5	1	9

* See footnote to Table 1

2-Carbamylphenylglucuronide This was isolated from salicylamide urine by the method described for 4-carbamylphenylglucuronide (Bray *et al.* 1947). The barium salt could not be obtained crystalline but the glucuronide itself crystallized readily. Yield 2 g from 12 g amide, m.p. 175–176° (decomp.), $[\alpha]_D^{25} - 84^\circ$ (water, c. 1). It did not reduce Benedict's solution (Found C, 49.8, H, 4.9, N, 4.3, glucuronic acid (after 5 min hydrolysis with 2N HCl) 59.02, equivalent (by titration) 315. $C_{13}H_{15}O_8N$ requires C, 49.9, H, 4.8, N, 4.5, glucuronic acid 62.0%, equivalent 313).

Attempted isolation of salicyluric acid (o-hydroxyhippuric acid) As already mentioned, there has been considerable disagreement about the conjugation of salicylic acid with glycine in animals, although it may be accepted that this occurs to some extent in man. We made several attempts to isolate salicyluric acid from rabbits' salicylate urine by fractionation of the ether soluble acid by means of various solvents (e.g. toluene, light petroleum), but in every case we were able to isolate only unchanged salicylic acid and hippuric acid. That the failure of

the rabbit to form salicyluric acid was not due to a lack of glycine in the diet was evident from the fact that we were still unable to isolate salicyluric acid from the urine of rabbits which had been given glycine (0.3 g) along with the salicylic acid (1 g). The reliability of our technique was shown by the ease with which we isolated salicyluric acid from the urine of children under salicylate therapy, using toluene for fractionation of the ether soluble acid. The product had m.p. 168–170° (mixed m.p. with hippuric acid 151°) and contained 6.79% N (Calc. for $C_9H_7O_4N$ N, 7.18%).

Isolation of metabolites of *m*-hydroxybenzoic acid and amide

Gentisic acid It is probable that this compound is a metabolite of both acid and amide. The $FeCl_3$ colour given by products isolated from hydrolyzed *m*-hydroxybenzoic acid and amide urines was deep blue, very similar to that given by gentisic acid, but it could not be assumed that this was the hydroxylation product, since 2,3-hydroxybenzoic acid, which is a theoretically possible metabolite, also gives a blue colour with $FeCl_3$ and has a similar m.p. (204°). The intensity of the colour given by products from *m*-hydroxybenzoic acid urine suggested that the amount formed was too small to encourage the hope of isolation. Attempts to isolate a dihydroxybenzoic acid from *m*-hydroxybenzamide urine were made by methods similar to that described in the previous section.

The urine excreted by 14 rabbits, each of which had received *m*-hydroxybenzamide (3 g), was hydrolyzed and extracted with ether as previously described. The ether soluble material thus obtained was dissolved in water, and the solution extracted with chloroform, which removed most of the *m*-hydroxybenzoic acid present. The aqueous solution was then evaporated to dryness, the residue dissolved in ether, and this solution extracted with $NaHCO_3$ solution. The extract was treated as for the isolation of gentisic acid. In this way more *m*-hydroxybenzoic acid, together with some hippuric acid, was crystallized. Recrystallization of the remaining solid from glacial acetic acid gave 100 mg colourless needles, m.p. 184–185°, which gave an intense blue colour with $FeCl_3$. Further recrystallizations from water failed to raise the m.p. to that of gentisic acid. Other attempts at isolation yielded similar products whether purification was by fractional crystallization or sublimation *in vacuo* at 140°. Analysis indicated the presence of about 1.6% N, an amount similar to that of the product obtained in one experiment from salicylamide (see above) (Found C, 57.3, H, 4.32, N, 1.6. Calc. for $C_7H_5O_4$ C, 54.5, H, 3.9%). We were unable to obtain a nitrogen-free product, even after a further period of heating with conc. HCl. That the substance

contained gentisic acid (or amide) is suggested by the fact that, on heating with pumice powder, a distillate was obtained which was identified as hydroquinone by mixed m p and mixed m p of the acetyl derivatives. Gentisic acid (or amide) is the only dihydroxybenzoic acid (or amide) which would give hydroquinone under these conditions. 2,3-Di-hydroxybenzoic acid would yield catechol. The distillate gave only the feeble fugitive blue-green colour with FeCl_3 typical of hydroquinone and no suggestion of the stable and definite colours given by catechol or resorcinol. We conclude, therefore, that gentisic acid (or amide) is a metabolite of *m*-hydroxybenzamide, although its isolation and positive identification is prevented by the formation of a nitrogen-containing complex.

3 Carbamylphenylglucuronide This compound was isolated by the method used for the other isomers. Crude barium salt (56 g) was obtained after the administration of 27 g *m*-hydroxybenzamide. Recrystallization gave a product m p 270° (decomp), $[\alpha]_D^{25} -59^\circ$ (water, c, 1) (Found N, 3.12. $\text{C}_{16}\text{H}_{15}\text{O}_8\text{N}_2\text{Ba}$ requires N, 3.35%). The glucuronide itself forms plates m p $163-164^\circ$ (decomp), $[\alpha]_D^{25} -67^\circ$ (water, c, 1). Yield 11 g (Found C, 49.1, H, 5.0, N, 4.3, glucuronic acid, 64.6, equivalent (by titration) 339. $\text{C}_{12}\text{H}_{12}\text{O}_6\text{N}$ requires C, 49.9, H, 4.8, N, 4.5, glucuronic acid, 62.0%, equivalent 313).

Attempted isolation of *m*-hydroxyhippuric acid We could not isolate this glycine conjugate from *m*-hydroxybenzoic acid urine by the method used successfully for the *p* isomer (Bray *et al* 1947). Unconjugated *m*-hydroxybenzoic acid and hippuric acid were readily isolated. We were still unable to isolate *m*-hydroxyhippuric acid after administering glycine (1 g) to the rabbit along with the *m*-hydroxybenzoic acid (3 g). It is of interest to note that *m*-hydroxyhippuric acid has not been claimed as a metabolite of *m*-hydroxybenzoic acid in the rabbit, but only in man and the dog. We have been unable to confirm the formation of this conjugate in man. Following Quick's (1932b) procedure, *m*-hydroxybenzoic acid and hippuric acid, but no *m*-hydroxyhippuric acid, were isolated from the urines of two human subjects who had each ingested *m*-hydroxybenzoic acid (3 g) with glycine (1 g). Our investigation of *m*-hydroxyhippuric acid was rendered more difficult by our failure to prepare it. The literature contains only two accounts of its preparation, in both cases from *m*-aminohippuric acid (Griess, 1868; Conrad, 1877). Neither provides unequivocal evidence for its existence. We were unable to obtain it also by Fischer's (1908, 1909) carbomethoxy method which is satisfactory for the *o* and *p* isomers. It should be noted that the claims of neither Baumann & Herter (1877-8) nor Quick (1932b) to have identified this compound as a meta-

bolite of *m*-hydroxybenzoic acid are supported by comparison with a synthetic specimen.

DISCUSSION

Table 4 summarizes the results of all our investigations of the metabolism of hydroxybenzoic acids and amides. It is evident that there are considerable differences between the fates of each acid and its amide. The degrees of hydrolysis of the amides are ortho, 4-7, meta, 4-16, para, 0-7%. These values are comparable with those obtained from *in vitro* studies with liver extracts (Bray, James, Ryman & Thorpe, 1948), i.e. ortho, 0, meta, 1, para, 6%. The presence of a hydroxyl group stabilizes the carbamyl group to a considerable extent, for benzamide itself is readily hydrolyzed to benzoic acid (Bray, Neale & Thorpe, 1946).

The investigation reported here raises several points of interest. The formation of gentisic acid as a hydroxylation product of salicylic acid and of gentisamide from salicylamide is in accordance with the general rule put forward in a previous paper (Bray, Lake, Neale, Thorpe & Wood, 1948). The fact that salicylic acid is hydroxylated to a greater extent than salicylamide is, however, unusual, since in most other cases, e.g. the aminobenzoic acids and amides, the opposite is true. A large number of so-called 'detoxication' reactions which foreign compounds undergo in the organism may be interpreted as occurring as a result of the formation of 'centres for conjugation' in the molecule, i.e. groups which can be conjugated with sulphuric, glucuronic and acetic acids, or with glycine. Thus, in compounds which already possess carboxyl and hydroxyl groups, conjugation is usually the principal mechanism, although a relatively small amount of hydroxylation occurs in some instances (e.g. *p*-hydroxybenzoic acid). If a 'potential' centre for conjugation is present, a possible reaction is its conversion to an 'actual' centre, e.g. the oxidation of a methyl group, as in acetotoluides (Jaffe & Hilbert, 1888; Bray & Thorpe, 1948), the hydrolysis of a carbamyl group to carboxyl as in benzamide (Bray *et al* 1946), or the reduction of an aromatic nitro group to amino as in *m*-nitrobenzaldehyde (Cohn, 1893). If, however, the 'potential' centre is not converted in this way, or converted to only a limited extent, e.g. as when the carbamyl group is stabilized by the presence of another group, as in the hydroxy- or amino benzamides (Bray, Lake, Neale, Thorpe & Wood, 1948), a new centre may be introduced by hydroxylation even if, as in *p*-hydroxybenzamide, one is already present though here the extent of hydroxylation is small. It was, therefore, unexpected to find that salicylamide was hydroxylated to a lesser extent than salicylic acid. The factor which determines the extent of these reactions is probably the

Table 4 *Metabolites of hydroxybenzoic acids and amides in rabbit*

Compound administered	Approx dose (g/kg)	Average percentage of dose excreted as				Approx % hydrolysis of amide
		Ether soluble acid	Ester glucuronide	Ether glucuronide	Ethereal sulphate	
<i>o</i> Hydroxybenzoic acid*	0.25	85	4	5	0	—
	0.5	85	3	14	0	—
<i>o</i> Hydroxybenzamide*	0.25	1	6	76	13	7
	0.5	0	4	67	16	4
<i>m</i> Hydroxybenzoic acid*	0.25	70	11	19	4	—
	0.5	84	7	11	2	—
	1.0	97	6	7	2	—
	1.5	97	6	2	2	—
<i>m</i> Hydroxybenzamide*	0.25	—	16	60	19	16†
	0.5	14	0	36	18	14
	1.0	10	1	28	11	11
	1.5	4	0	25	10	4
<i>p</i> Hydroxybenzoic acid‡	0.1	66	16	19	4	—
	0.25	65	5	10	7	—
	0.5	75	4	6	7	—
	1.0	73	6	12	5	—
	1.5	64	7	8	5	—
<i>p</i> Hydroxybenzamide‡	0.1	2	0	15	35	2
	0.25	0	0	15	23	0
	0.5	7	0	15	17	7
	1.0	3	1	24	17	4
	1.5	5	1	22	14	6

* Diet pellets

† This value is low since it does not include ether soluble acid excretion

‡ Diet bran, oats and cabbage (Bray *et al* 1947)

availability of suitable enzyme systems to which the compound under consideration is acceptable. It would seem that the configuration of salicylamide is even less acceptable to the enzyme responsible for hydroxylation than is salicylic acid. It may be noted that *o* substituted compounds are often metabolized to a smaller extent than the corresponding *m*- and *p* isomers (e.g. sulphate conjugation of hydroxybenzoic acids, acetylation of aminobenzoic acids). The formation of gentisic acid as a hydroxylation product of *m* hydroxybenzoic acid or its amide would be in accordance with our general rule.

With regard to the nature of the hydroxylation products derived from the hydroxybenzoic acids and amides it is of interest to refer to an investigation of Dakin & Herter (1907). It is well known that hydrogen peroxide under various conditions can hydroxylate benzene derivatives, giving in many cases products identical with those formed as metabolites of the original compound, e.g. benzene gives phenol, catechol and hydroquinone (e.g. Cross, Bevan & Heiberg, 1900; Baernstein, 1946). Dakin & Herter found that hydrogen peroxide oxidized ammonium salicylate to 2,3-dihydroxybenzoic acid and the ammonium salts of both the *m*- and *p* isomers to protocatechuic acid (3,4-dihydroxybenzoic acid). (In spite of several attempts we failed to obtain 2,3-dihydroxybenzoic acid in this way, although we had no difficulty in isolating protocatechuic acid by oxidizing *m*- or *p* hydroxybenzoic

acid using Dakin & Herter's directions.) 2,3-Dihydroxybenzoic acid has not been reported as a metabolite of salicylic acid, and we were unable to detect the formation of protocatechuic acid from *m* hydroxybenzoic acid, although we showed it to be a metabolite of both *p* hydroxybenzoic acid and its amide (Bray *et al* 1947). We are also investigating the effect of hydrogen peroxide on the hydroxybenzamides, and hope to extend the study to a wider comparison of *in vitro* and *in vivo* hydroxylation.

SUMMARY

1 The metabolism of *o* and *m* hydroxybenzoic acids and their amides in the rabbit has been investigated.

2 A large proportion (85%) of salicylic acid is excreted as ether soluble acid, 3–4% as ester glucuronide and 5–14% as ether glucuronide. The corresponding values for salicylamide are 0–1, 4–6 and 67–76%. Salicylic acid does not affect ethereal sulphate excretion, but 13–16% of salicylamide is excreted conjugated with sulphuric acid.

3 The percentages of *m* hydroxybenzoic acid excreted as ether soluble acid, ester glucuronide, ether glucuronide and ethereal sulphate are 70–97, 6–11, 2–19 and 2–4 respectively. The corresponding values for *m* hydroxybenzamide are 4–14, 0–16, 25–60 and 10–19%.

4 From the above results it is calculated that the degree of hydrolysis of the carbamyl group of salicylamide is 4-7 and of *m* hydroxybenzamide 4-16%

5 2 and 3 carbamylphenylglucuronides have been isolated as major metabolites of salicylamide and *m* hydroxybenzamide respectively

6 Gentisic acid (4-5%) has been isolated as a metabolite of salicylic acid and gentisamide of salicylamide

7 Evidence has been obtained of the formation of gentisic acid (or amide) as a metabolite of *m* hydroxybenzoic acid and amide

8 Glycine conjugates of *o* and *m* hydroxybenzoic acids were not detected

9 The fact that salicylic acid is hydroxylated to an extent greater than is salicylamide is discussed

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Growth Factors for *Lactobacillus casei*

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We have described elsewhere (Chattaway, Happold, Lythgoe, Sandford & Todd, 1943; Chattaway, Happold & Sandford, 1944) the presence of a growth factor for *Corynebacterium diphtheriae gravis*, sub-type Dundee, in the amyl alcohol insoluble fraction obtained by extracting liver residues with this solvent at pH 2, and the separation of this factor from material stimulating the growth of *Lactobacillus casei* at pH 3, the *Lb. casei* factors being in the cresol extract

The present paper describes the further concentration and separation of the factors promoting acid production by *Lb. casei* (Chattaway, Dolby & Happold, 1944), and Factor 3 of Dolby, Happold & Sandford (1944), and considers their relationships to

factors reported as stimulatory for this organism by other workers. Among these factors are fractions from liver, fermentation residues and yeast, which have been shown to be members of the pteroyl-glutamic acid (PGA) group of components, differing from one another in having one, three or seven glutamyl residues respectively (Angier, Boothe, Cosulich, Fahrenbach, Hultquist, Hutchings, Kuh, Mowat, Northey, Seager, Semb, Sickels, Smith, Stokstad, Subbarow & Waller, 1945, 1946; Bloom, Calkins, O'Dell & Piffner, 1946). The material known as folic acid on the other hand (Mitchell, Snell & Williams, 1941) is probably different from these, as also is the '*Streptococcus lactis* R' factor (Keresztesy, Riches & Stokes, 1943) which has been shown

to be 4-*N* formyl *N*-(2'-amino-4'-hydroxyl-6'-pteridylmethyl) aminobenzoic acid (Anderson, Arth, Folkers, Harris, Kaczka, Mozingo, Southwick & Wolf, 1947), but it has been claimed (Angier *et al* 1946) that PGA represents the sole remaining unknown growth factor for *Lb casei*. The following observations by various workers indicate that this is probably not so

(1) Williams (1944) has stated that folic acid probably consists of a number of closely related compounds with differing physiological activity, a statement supported by Hall (1947*a*) in this Department

(2) Mitchell, Snell & Williams (1944) reported the absorption spectrum of folic acid as indicating the presence of a xanthopterin nucleus, while that of PGA does not

(3) Hall (1947*b*) has demonstrated differences in the response of *Strep faecalis* R to folic acid, vitamin B₉ and PGA.

(4) Elvehjem & Teply (1945) found the need for unidentified factors other than vitamin B₉ in the growth of *Lb casei*

(5) Dolby *et al* (1944) showed the *C diphtheriae* factor to be present in casein and to be required for the growth of *Lb casei*. They also reported the presence of a growth factor (Factor 2) distinct from the *C diphtheriae* factor, which did not affect the amount of acid production by *Lb casei*, but which did increase its growth. It appears to us possible that this factor might be related to the streptogenin factor of Woolley (1941)

(6) Barton-Wright, Emery & Robinson (1944, 1945) describe two chloroform soluble fractions obtained from liver which are synergistic for *Lb casei* and not identical with folic acid.

Evidence will be presented in this paper to show that on our basal medium the addition of either PGA or 'folic acid' (7% concn kindly supplied by Prof R J Williams) produces little response, either in the growth or acid production of *Lb casei*, thus demonstrating the need of this organism for further growth factors

EXPERIMENTAL

Organisms and media The medium used for investigation with *Lb casei* has been previously described (Chattaway, Happold & Sandford, 1943), the techniques of culture and subculture were unchanged. Readings were made after 72 hr and recorded as ml. 0.1N acid produced

In the determination of 'folic acid' activity, *Strep faecalis* R was used, the medium employed was that of Gurard, Snell & Williams (1942) with the addition of leucine and isoleucine (of Dolby & Waters, 1944), and using an acid hydrolysate of casein prepared according to the method of Johnson & Mueller (1941). Acid production was determined after 18 hr and recorded as ml. 0.05N acid produced

Preliminary concentration of active material The starting material was that fraction from the liver residues (Boots Pure Drug Co., Ltd., Nottingham) which was not adsorbed on fuller's earth at pH 3, but which was subsequently adsorbed on norite at this pH, and then eluted with an ammonia ethanol water mixture (containing 12% (w/v)

NH₃ in 50% (v/v) ethanol). The eluate was neutralized, concentrated, acidified to pH 2 and extracted repeatedly with amyl alcohol. The amyl alcohol insoluble material (water soluble) was then extracted with *p*-cresol at pH 3, and the cresol extract used for the work described below

RESULTS

Separation of two synergistic fractions by treatment with baryta

Active concentrates prepared in the above manner gave a considerable precipitate on saturation with baryta, additional quantities being obtained by the addition of 3 volumes methylated spirits to the baryta saturated material. The baryta soluble material was found, after removal of excess barium, to show little loss in activity compared with the original material when tested with *Lb casei*. The insoluble barium salts, after removal of barium, showed little intrinsic activity, but when the two fractions were tested together at low concentrations it became apparent that the original activity was due to two components. A typical set of results is shown in Table 1

Table 1 *Acid production by cultures of Lactobacillus casei on inclusion of baryta-soluble and insoluble fractions*

Additions (ml/20 ml. medium)		Acid production after 72 hr (ml 0.1N acid/20 ml. medium)
Baryta soluble	Baryta insoluble	
1.0	—	6.3
0.5	—	5.2
0.05	—	2.6
0.005	—	1.1
0.0005	—	0.2
—	1.0	1.4
—	0.3	0.9
—	0.1	0.6
—	0.01	0.2
—	0.001	Nil
0.01	0.01	4.4
0.001	0.01	2.9
0.0001	0.01	1.8
—	—	Nil

Fractionation of the baryta-soluble material

(a) **Phosphotungstic acid** The material was treated with excess of 50% (w/v) phosphotungstic acid in 5% (w/v) H₂SO₄, the precipitate removed and the filtrate freed from phosphotungstic acid with Ba(OH)₂. There was some loss of activity (20–30%) in the process, but practically the whole of the remaining activity was in this filtrate

(b) **Picric acid** On treatment of the Ba(OH)₂ soluble material with saturated picric acid, small amounts of a picrate separated which always showed slight activity, yet there was no detectable loss of activity in the soluble fraction after removal of excess picric acid by ether extraction.

(c) *Silver nitrate*. Treatment of the $\text{Ba}(\text{OH})_2$ soluble fraction with AgNO_3 at acid reactions effected a considerable fractionation of the active material, the following technique being adopted. The solution was acidified with excess HCl in the cold and NaCl , free HCl and silver salts of some organic acids were removed by stepwise addition of solid AgNO_3 (ppt 1), the reaction remaining at pH 1.0 (approx.) An excess of AgNO_3 was then added and the reaction adjusted cautiously to pH 4.0 with NaOH , a flocculent precipitate, which later darkened, formed and was removed (ppt 2). With further additions of NaOH a white, heavier precipitate (ppt 3) which was inactive began to appear at pH 4.5, increasing in amount at more alkaline reaction. The whole activity of the $\text{Ba}(\text{OH})_2$ soluble material for *Lb casei* was found in ppt 2, the active material was recovered by treatment with a mixed solution of saline and HCl carefully adjusted to give a neutral supernatant readily separated from the precipitated AgCl . The 'folic acid' activity measured on cultures of *Strep faecalis* R was concentrated mainly in ppt 1, although some remained in ppt 2. There was no measurable activity in ppt 3 and later residue fractions.

On addition of ethanol (50 to 60%, v/v) to the aqueous solution of ppt 2 an inactive white crystalline precipitate separated at 0°. Addition of ether (50%, v/v) gave a further separation of white inactive material in the ethanol phase. From the ether water phase, which was orange in colour, a reddish brown oil began to separate, the activity being distributed between these two components.

(d) *Lead acetate*. Attempts to fractionate the $\text{Ba}(\text{OH})_2$ soluble material by treatment with lead acetate at pH 3 followed by basic lead acetate at pH 7.6 were unsuccessful. The active material was found to be unevenly and variably divided between the precipitates and some slight activity remained in the filtrate.

(e) *Adsorption on alumina*. The alumina used was 'Alumina purified for chromatographic purposes' (British Drug Houses Ltd.). It was suspended in 40% methanol at pH 3 and kept at 37° for 3 days, filtered, air-dried at room temperature for 4 days, suspended in 40% methanol at pH 3 and poured into a column 30 cm by 2.5 cm. The column so prepared was washed through with 40% methanol at pH 3 using approximately 250 ml. A slight positive pressure of N_2 was maintained on the column during this preliminary washing and during the subsequent run with the active material. The material to be treated, dissolved in 40% methanol at pH 3, was added to the top of the column, and washed through with acidified 40% methanol and the filtrate collected until quite colourless and inactive for *Lb casei*. Elution was carried out using 2% (w/v) NH_3 . Filtrate and eluate fractions were collected and concentrated *in vacuo* as soon as possible, dissolved in a small volume of water and adjusted to pH 6.8.

The usual procedure adopted with the baryta soluble material was to separate it into filtrate and 2% (w/v) NH_3 eluate fractions as described above, and then to pass both of these fractions, separately, down further alumina columns, finally combining all filtrate fractions and all eluate fractions. All fractions were tested for acid producing activity using *Lb casei* and *Strep faecalis* R. The filtrate fractions were found to be almost inactive while the eluate fraction contained considerable active material for *Strep faecalis* R, indicating that this material probably contains some or all of the components of 'folic acid' (Mitchell *et al.* 1944). Both

fractions contained material active for *Lb casei* and they were shown to be synergistic in their action. Typical results are shown in Tables 2 and 3.

Table 2 Separation of baryta soluble material into two active fractions by treatment with alumina

(Baryta-insoluble fraction 0.01 ml. was added to all flasks in *Lb casei* tests. Concentrations tested Al_2O_3 filtrate fraction, 310 mg/ml., Al_2O_3 eluate fraction, 153 mg/ml.)

Al_2O_3 filtrate (ml./20 ml. culture)	Al_2O_3 eluate (ml./20 ml. culture)	Acid production	
		<i>Lb casei</i> (ml 0.1 N- acid/20 ml. medium, 72 hr incubation)	<i>Strep faecalis</i> R (ml 0.05 N acid/10 ml. medium, 18 hr incubation)
1.0	—	7.25	1.2
0.1	—	3.45	0.25
0.01	—	2.25	Nil
0.001	—	1.7	Nil
—	1.0	4.45	—
—	0.1	2.5	—
—	0.01	1.5	4.6
—	0.001	—	3.05
—	0.0001	—	0.95
Nil	Nil	1.4	Nil

Table 3 Synergism between Al_2O_3 -filtrate and eluate fractions for *Lactobacillus casei*

(No lactic acid was produced when fractions were tested individually as follows in presence of baryta insoluble fraction Al_2O_3 filtrate, 0.01, 0.001, 0.0001 ml., Al_2O_3 eluate, 0.01, 0.001 ml. All figures refer to quantities/20 ml. medium and 72 hr incubation.)

Al_2O_3 filtrate (ml.)	Al_2O_3 eluate (ml.)	0.1 N acid (ml.)
0.01	0.01	1.7
0.01	0.001	1.65
0.005	0.005	1.3
0.005	0.0005	1.05
0.0001	0.001	0.15
0.00005	0.0005	Nil
Nil	Nil	Nil

Fractionation of material from silver precipitate 2 in alumina column

The orange-yellow pigment and the reddish-brown oil obtained from precipitate 2 (see above) were bulked, and examined by chromatography on Al_2O_3 after solution in 40% (v/v) methanol at pH 3.0. As each fraction was collected it was concentrated to dryness *in vacuo*, and redissolved in water. The fractions were all tested several times with *Lb casei* on the basal medium, with and without both pteroylglutamic acid and the baryta insoluble fraction at the lower limit of its activity. They were tested for 'folic acid' activity with *Strep faecalis* R. The results are not completely conclusive, but Table 4, which shows the treatment each fraction received, also gives a summary (columns 4 and 5) of their activity for both the organisms employed.

Table 4 *Activities of fractions produced from the pigmented fractions of the silver separation by chromatography on alumina*(None of the fractions showed any activity for *Lb casei* when administered alone or with the baryta insoluble fraction)

No of fraction	Colour	Treatment producing fraction	Activity	
			<i>Strep faecalis</i> R	<i>Lb casei</i> with PGA
I	Pale yellow	1st filtrate fraction	Equivalent to 0.001 µg PGA/ml	Slight activity
II	Colourless	2nd filtrate fraction	Inactive	Active
III	Pale yellow	1st eluate, 20% (v/v) methanol	Inactive changing to active	Growth, but no acid
IV	Pale yellow	2nd eluate, water for 12 hr	Slightly active	Inactive
V	Colourless	3rd eluate, 0.5% (w/v) NH ₃ collected prior to appearance of first pigmented band	Inactive	Inactive
VI	Dark brown	4th eluate, 0.5% (w/v) NH ₃ , pH of fraction 6.8-7.0	Active	Very active
VII	Dark brown	5th eluate, 0.5% (w/v) NH ₃ , pH 7.2 upwards	Active approx \equiv 1 µg PGA/ml	Very active
VIII	Pale yellow	6th eluate obtained after 6 hr washing with 0.5% (w/v) NH ₃	Active	Slightly active

These fractions were tested again later and fraction III, which had previously been inactive, had acquired measurable activity, so possibly some 'folic acid-like' component had slowly hydrolyzed from a more complex peptide. In the cultures where the basal medium had aged somewhat, and where the controls were consequently positive—a finding previously reported by us and explicable on the basis of the findings of Hall (1947c) in this laboratory that 'folic acid like' activity develops in amino acid sugar media containing histidine—the above fractions, which in fresh media gave growth and acidity only in the presence of PGA, did so without the addition of this component.

These results suggest the presence in the filtrate fraction of an active component for *Lb casei* which is probably not the yellow substance present since activity is measurably greater in the colourless fraction II. Another active component is present in association with the pigments present in fractions VI and VII. Attempts to show synergistic action between fractions I-VII were not very successful though the critical interpretation of such experiments at each stage in an investigation is often difficult. The possibility must not be overlooked, however, that the same or similar compounds may be combined with other groups, in say, fractions II or VI and VII, which would modify their behaviour on the alumina.

In previous work, with repeated ethanol ether precipitations from a silver salt similar to precipitate 2, a fraction had been obtained which it was expected would correspond roughly to fraction VII. This material was similarly developed upon an Al₂O₃ chromatogram. The colourless filtrate fraction was inadvertently thrown away, but a colourless water eluate A (cf fraction IV, Table 4), a colourless

fraction B (cf fraction V) after treatment with 0.5% (w/v) NH₃, the main pigment, fraction C (pH 6.8-7.0, cf fraction VII), and the colourless eluate D were obtained. Fraction C was the only one to give unequivocal positive results with *Lb casei*, but its activity was increased by fraction D although this fraction alone was inactive. These results suggest that more than one component is present in the alumina eluate fraction from the baryta soluble material.

Properties of Al₂O₃ filtrate and eluate fractions

The effect of the following reagents on the activity of the above fractions for *Lb casei* and *Strep faecalis* R was determined, the filtrate fraction being tested with *Lb casei* only and the eluate fraction with both organisms.

Table 5 *Effect of nitrous acid treatment of Al₂O₃-eluate fraction, tested with Lactobacillus casei*

Material added	(ml./20 ml medium)	Acid production in 72 hr (ml. 0.1 N acid)
Al ₂ O ₃ eluate	0.1	3.8
	0.075	4.5
	0.05	2.6
HNO ₂ treated Al ₂ O ₃ eluate	0.1	1.5
	0.075	1.4
	0.05	1.1
HCl treated Al ₂ O ₃ eluate	0.1	3.6
	0.075	4.0
	0.05	2.5

(a) *Nitrous acid* 0.5 ml of each of the fractions was taken, together with 10 ml distilled water, 40 mg NaNO₂ and 1 ml dilute HCl and the solution left to stand for 24 hr at room temperature, evaporated to dryness, redissolved in

water and adjusted to pH 7 (volume to 25 ml) A control was carried out omitting the NaNO_2 . Some inhibitory substance is obviously present in the Al_2O_3 eluate which is apparent in the highest concentration of eluate used. The Al_2O_3 filtrate fraction showed no inactivation while the eluate fraction showed partial inactivation for *Lb. casei* (see Table 5), and complete inactivation for *Strep. faecalis* R. These results accord with the previous observation that at least two components are present in the eluate fraction.

(b) *Acetic anhydride* To 1 ml. fraction were added 3.2 ml. 2N NaOH and 0.2 ml. acetic anhydride. The mixture was shaken and cooled in ice. Five further additions of 2 ml.

material being removed from the control by chloroform. However, when tested against *Lb. casei* the results were difficult to assess, as components active for this organism were removed by the chloroform extraction.

(d) *Methanolic HCl* The solid residue from 0.5 ml. of each fraction was refluxed for 4 hr. with dry methanol saturated with dry HCl. Excess reagent was removed *in vacuo*, and the residue dissolved in water, adjusted to pH 7, and made up to 25 ml. The activity for *Lb. casei* was almost completely destroyed by the treatment and attempts to regenerate it by hydrolysis were unsuccessful. A summary of the above results is given in Table 7.

Table 6 *Effect of acetylation of Al_2O_3 eluate fraction on acid production by Streptococcus faecalis R*

Al_2O_3 -eluate fraction (ml / 10 ml medium)	Acid production (ml 0.05N acid)		Acetylated Al_2O_3 eluate fraction (ml / 10 ml medium)	Acid production (ml 0.05N acid)	
	16 hr incubation	72 hr incubation		16 hr incubation	72 hr incubation
0.02	4.1	9.75	0.02	Nil	9.3
0.01	3.9	9.7	0.01	0.4	8.1
0.003	2.9	8.45	0.003	1.5	6.5
0.001	2.2	7.2	0.001	0.9	4.85
0.0003	1.1	5.5	0.0003	0.25	3.4
0.0001	0.4	4.0	0.0001	Nil	2.65
Basal medium	Nil	2.7	—	—	—

Table 7 *Effect of various reagents on the activity of fractions from the baryta soluble material*

Reagent	Al_2O_3 filtrate <i>Lb. casei</i>	Al_2O_3 eluate	
		<i>Lb. casei</i>	<i>Strep. faecalis</i> R
HNO_3	Full activity	Partial loss	100% loss
Acetic anhydride	100% loss	? little loss	No loss
Ninhydrin	Indeterminable	Indeterminable	25% loss
Methanolic HCl	100% loss	100% loss	—

2N NaOH and 0.2 ml. acetic anhydride were made at 1 min. intervals. After 10 min. at room temperature the mixture was acidified with H_2SO_4 to pH 6.8 and diluted to a final volume of 15 ml. The Al_2O_3 filtrate fraction was completely inactivated by this treatment. The eluate fraction, tested against *Strep. faecalis* R, was apparently inactive if the cultures were examined after 16 hr. incubation, but the organism could slowly utilize the acetylated material which, at 72 hr. incubation, was almost as active as the original material (see Table 6). By an oversight, the acetylated eluate fraction was not tested with *Lb. casei*, but crude preparations so treated have not shown any great loss of activity, and since growth is continued for 72 hr. with this organism, it is likely that no great inactivation would have been demonstrated.

(c) *Ninhydrin* 10 ml. of one third saturated KH_2PO_4 solution were added to 0.5 ml. of each fraction and 120 mg. ninhydrin. After boiling for 15 min. sufficient alanine was added to react with excess ninhydrin. The mixture was again boiled, extracted with chloroform to remove the coloured products, the insoluble material centrifuged off and the aqueous solution adjusted to pH 6.8 and diluted to 25 ml. 0.5 ml. of the same fraction in 10 ml. of the phosphate solution was extracted with chloroform and used as a control. The Al_2O_3 filtrate showed 80% inactivation with ninhydrin, no active material being extracted from the control by chloroform. The eluate fraction tested against *Strep. faecalis* R showed 25% inactivation, no active

Fractionation of the baryta insoluble material

At pH 4 a large proportion of the active material could be extracted from aqueous solution by chloroform. Some active material was extracted at pH 7 which appeared to be similar to that extracted at pH 4. Portions of this material, prior to chloroform extraction, were treated with the same reagents as the baryta soluble fractions, the results being shown in Table 8.

Table 8 *Effect of various reagents on baryta insoluble material*

Reagent	Effect on activity	
	<i>Lb. casei</i>	<i>Strep. faecalis</i> R
HNO_3	100% loss	100% loss
Acetic anhydride	100% loss	100% loss
Ninhydrin	Full activity	Full activity
Methanolic HCl	100% loss	100% loss

Response of *Lactobacillus casei* to baryta soluble fraction, pteroylglutamic acid and 'folc acid'

The three materials were tested separately, and PGA and the baryta soluble fraction were also examined for synergism. Typical results are shown

in Table 9 Neither PGA nor folic acid tested on our medium show an activity comparable with that reported by Anger *et al* (1945), and Mitchell *et al* (1941), since PGA is claimed to be 50 % active in 1×10^{-6} μg /ml medium. This fact and the higher titrations obtained with the baryta-soluble fraction demonstrate the existence of unidentified factors essential for the growth of *Lb casei* on purified media. There is some evidence for synergism between PGA and the baryta-soluble fraction, but this effect was not expected to be very pronounced as the latter contained traces of PGA.

Table 9 *Effect of baryta soluble fraction, pteroyl glutamic acid and folic acid on acid production by Lactobacillus casei*

PGA (1×10^{-3} μg / ml. medium)	Folic acid (μg /ml. medium)	Baryta soluble fraction (1×10^{-3} ml./ ml. medium)	Acid production after 72 hr incubation (ml 0.1 N acid/20 ml medium)
—	—	—	Nil
—	—	2.50	4.9
—	—	0.025	2.0
5.0	—	—	2.9
0.50	—	—	2.0
0.05	—	—	0.3
0.005	—	—	0.4
—	0.6	—	0.7
—	0.1	—	0.6
—	0.01	—	0.2
5.0	—	2.50	6.6
5.0	—	0.025	2.4
0.05	—	2.50	5.9
0.05	—	0.025	2.8

DISCUSSION

There are considerable discrepancies in the claims made as to the growth requirements of *Lb casei*. The variations in basal media used in different laboratories may account for many of these differences. It is generally recognized that such materials as casein and peptone contain quantities of vitamins sufficient to affect micro organisms, and no standard procedure is adopted for the removal of these contaminants. In particular, emphasis may be placed on the methods of preparation of casein digests. In these laboratories, digestion is carried out with hydrochloric acid followed by precipitation with litharge to pH 4 and then with barium sulphide. It can be demonstrated that most of the *Lb casei*-active material is removed from liver concentrates in lead precipitates at this pH. Several groups of American workers, however, prepare a digest with sulphuric acid followed by neutralization with baryta, a process which does not remove appreciable *Lb casei* activity from liver fractions.

Using our casein digest as the medium for the experiments, the growth factors present in liver extract, additional to the *C diphtheriae* factor and the chloroform-soluble factors of Barton-Wright *et*

al (1944), have been separated into the following components: (1) a factor or factors insoluble in saturated baryta, and (2) at least three factors soluble in saturated baryta and in silver nitrate at pH 1.0—a filtrate fraction which is not adsorbed on alumina at pH 3, and two fractions which are adsorbed, one of which is eluted with 20 % (v/v) methanol or water and the other with 0.5 % (w/v) ammonia. These three components have properties dissimilar to both PGA and the folic acid of Mitchell *et al* (1941). The greatest concentration of folic acid-like material was found in the silver salts insoluble at pH 1 (from the baryta soluble material) which contained none of the components discussed above.

The differential action of ninhydrin and nitrous acid, and of esterification and acetylation upon the activity of the 2 % (w/v) ammonia eluate material for *Strep faecalis* R and *Lb casei*, confirms that there are two components present in this material. The fact that the above chemical treatment affects the filtrate fraction and the baryta insoluble fraction dissimilarly to one another and to the above ammonia eluate material is evidence that we have four separate and distinct factors.

The possibility that folic acid-like compounds might be split during fractionation from more complex components, which themselves show no activity immediately on separation, complicated studies such as these, and, indeed, there is some evidence to support such a view. This does not, however, invalidate the claim that several active and synergistic compounds are present in the starting material. The difficulty of obtaining adequate supplies of material, which had caused work to cease on these fractions for over two years, makes the publication of these results desirable.

SUMMARY

1 The presence is demonstrated of further unidentified growth factors for *Lactobacillus casei*, present in liver.

2 The crude material contains a factor or factors insoluble in baryta, and at least three factors soluble in saturated baryta.

3 The baryta soluble factors may be further separated by treatment with alumina, two components are adsorbed and may be separated by fractional elution, and one is present in the filtrate.

4 The effect of a number of chemical reagents on the activity of these components has been studied and shown to distinguish them from the pteroyl-glutamic acid group of factors and from folic acid.

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Lipids of Normal Brain

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In most of the early studies on the distribution of the lipids in the brain large samples of tissue were used, and the various fractions isolated, more or less quantitatively, by the classical differential solubility techniques. Extensive reviews of this work have been given by Page (1937) and Bloor (1943).

To investigate experimentally degenerative conditions in the nervous system, methods must be available which require only small samples of tissue. In recent years many such micro methods have been reported. In the experiments described in this paper the micro methods at present in use in this laboratory have been applied to normal brain tissue.

METHODS

Reagents. All reagents used were of A.R. grade, and all solvents were freshly redistilled on the day of use.

Extraction of lipids

The brain was dissected out as soon as possible after the animal had been killed. A small sample of tissue (1-2 g) was rapidly weighed, and extracted by a modification of the

method of Bloor (1929). It was ground in a mortar with a small quantity of acid washed, ether-extracted sand, and successively extracted with 50 ml ethanol (2 portions), 50 ml 1:1 ethanol-ether mixture (8 portions) and 50 ml ether (1 portion). Each extract was filtered in turn through an ether-extracted Whatman no. 2 filter paper into a 500 ml volumetric flask, and the contents of the flask finally made up to volume with ether. By this procedure, in which the proportion of solvent to tissue was greater than that usually adopted, the lipids were completely extracted from brain tissue. To verify this, the insoluble residue was extracted for 6 hr with ether in a continuous extraction apparatus, followed by a further 6 hr with methanol. The combined extract was found to contain neither cerebroside nor cholesterol and only negligible quantities of phospholipid. When the same procedure was applied to liver tissue, however, it was found that a considerable phospholipid fraction was not extracted.

Three separate 100 ml samples of the ether-ethanol extract were placed in a 60° water bath, and evaporated just to dryness under reduced pressure and in an atmosphere of N₂. The residue was extracted successively with eight 2 ml portions of light petroleum (b.p. 40-60°) and the light petroleum evaporated down to 1 ml on a 60° water bath. The phospholipids were precipitated by adding 30 ml

acetone and 20 drops of a saturated solution of $MgCl_2$ in ethanol. After centrifuging, the clear supernatant liquid was decanted into a 50 ml volumetric flask. The ppt was washed with 10 ml acetone and the acetone washings were added to the 50 ml volumetric flask which was then made up to volume with acetone. The ppt was dissolved in 5 ml of a 1:1 methanol-ether mixture, and transferred quantitatively to a 25 ml volumetric flask which was made up to volume with 1:1 methanol-ether mixture.

All analyses were done in triplicate, the sample for the cerebroside estimation being taken from the original ethanol-ether extract, those for the cholesterol from each of the three 50 ml volumetric flasks containing the acetone soluble material, and those for the phospholipids from each of the three 25 ml volumetric flasks containing the methanol-ether solution.

Free and total cholesterol were estimated in 2 ml of the acetone solution by a modification of the method of Schoenheimer & Sperry (1934), incorporating improvements suggested by Sperry & Brand (1943) and Sobel & Mayer (1945). The colour was read in a Coleman Universal spectrophotometer at 620 $m\mu$.

Cerebrosides were estimated by the method of Brand & Sperry (1941) in which the galactose liberated on hydrolysis is estimated by the ceric sulphate titration procedure of Miller & Van Slyke (1936). Six 25 ml samples were taken from the original ethanol-ether extract and, of these, three were hydrolyzed, and three treated in exactly the same way, except that the hydrolysis was omitted. Eriogreen proved to be a more satisfactory indicator than either setopaline C or the o-phenanthroline ferrous complex suggested by Macy (1942). It was found that more reproducible results could be obtained if the unhydrolyzed as well as the hydrolyzed sample was neutralized to chlorophenol red before the clarification procedure.

Phospholipids The procedure adopted for the determination of the various phospholipids was the same as that described by Haack (1947). It depends on the observation that the monoaminophospholipids, lecithin and cephalin, are completely hydrolyzed by N KOH in 18 hr at 37° whereas, under these conditions, there is no hydrolysis of sphingomyelin. The total acid soluble P in the hydrolysis mixture is a measure of the lecithin and cephalin present (Schmidt, Benotti, Hershman & Thannhauser, 1946) and the choline a measure of the lecithin (Haack, 1947). The difference between the total P and the total acid soluble P in the hydrolysate is a measure of the sphingomyelin, and the difference between the molecular equivalents of monoaminophospholipid and choline gives the molecular equivalents of cephalin.

For the estimation of total lipid P, 1 ml was taken from the 25 ml volumetric flask containing the methanol-ether solution of the phospholipids, and the total P was determined by the method of Fiske & Subbarow (1925). The phospholipid was calculated on the basis of

$$\text{total phospholipid} = \text{total lipid P} \times 25$$

The monoaminophospholipid was estimated in the remaining 24 ml of the methanol-ether solution which was evaporated under reduced pressure at 60° to a volume of 0.2 ml. After the addition of 5 ml N KOH, the tube was stoppered and shaken at 37° for 18 hr. To 0.5 ml of this solution, pipetted into a centrifuge tube, there were added 0.1 ml 5*N* HCl and 3 ml 10% (w/v) trichloroacetic acid.

The centrifuge tube was stoppered, shaken, and kept for 1–2 hr at room temperature. After centrifuging, the solution was filtered through a Whatman no. 44 filter paper, and the total P estimated in 2 ml of the trichloroacetic acid filtrate (representing 1.28 ml of the methanol ether solution) by the method of Fiske & Subbarow (1925). After correction had been made for the various dilutions, the monoaminophospholipid was calculated on the basis of

$$\text{monoaminophospholipid} = \text{total P of} \\ \text{trichloroacetic acid filtrate} \times 25$$

An estimate of the lecithin was obtained by determining the choline in the hydrolysate by what was essentially the method of Glick (1944). To the remainder of the hydrolysate (equivalent to 21.7 ml of the methanol ether solution) there was added 1 drop of 1% (w/v) thymolphthalein in ethanol, followed by glacial acetic acid, drop by drop, until the blue colour disappeared. The solution was filtered through a Whatman no. 44 filter paper, and washed with three 2 ml portions of distilled water.

To the filtrate was added 2 ml 2% (w/v) ammonium reineckate in methanol, and the tube kept in the refrigerator overnight for the precipitation of the choline. The remainder of the procedure was carried out as described by Glick (1944), except that the choline reineckate was dissolved in a final volume of 4 ml acetone, and the colour density measured in a Coleman Universal spectrophotometer at 526 $m\mu$. A standard solution containing 500 μg choline chloride was taken through at the same time. After correction had been made for the various dilutions, the lecithin was calculated on the basis of

$$\text{lecithin} = \text{choline (as choline chloride) in the} \\ \text{trichloroacetic acid filtrate} \times 5.56$$

The sphingomyelin and cephalin were calculated as follows

$$\begin{aligned} \text{sphingomyelin} &= \text{total phospholipid} \\ &\quad - \text{monoaminophospholipid} \\ \text{cephalin} &= \text{monoaminophospholipid} - \text{lecithin} \end{aligned}$$

RESULTS

The concentration of cerebroside, cholesterol and phospholipid in guinea pig, cat and rabbit brain is given in Table 1, and Table 2 gives the concentration of the individual phospholipids, lecithin, sphingomyelin and cephalin. In the experiments reported in these two tables the investigations were done on whole brain, no attempt being made to separate grey matter from white matter. It will be seen that the distribution pattern of the lipids was similar for each species studied.

Table 3 shows the cerebroside, cholesterol and phospholipid concentration in both grey matter and white matter from brains of the cat, dog, beaver and man, while Table 4 shows the concentration of each of the individual phospholipid fractions which constitute the total phospholipids.

The figures of Tables 1–4 are in terms of mg/100 mg fresh tissue. It is seen that, on this basis, there was a greater concentration of cerebroside, total cholesterol, and total phospholipid in the white matter than in the grey for every species studied.

Table 1 *Lipids in normal brain, expressed as mg /100 mg fresh tissue (mean \pm s.e. mean)*

	No of animals	Cerebroside	Free cholesterol	Total cholesterol	Ester cholesterol	Total phospholipids
Guinea pig	8	2 29 \pm 0 13	1 81 \pm 0 09	1 76 \pm 0 10	0 15 \pm 0 07	4 82 \pm 0 28
Cat	8	1 88 \pm 0 12	1 89 \pm 0 07	1 94 \pm 0 07	0 05 \pm 0 02	4 57 \pm 0 29
Rabbit	4	2 74 \pm 0 14	2 16 \pm 0 06	2 21 \pm 0 06	0 05 \pm 0 02	5 40 \pm 0 18

Table 2 *Phospholipids in normal brain, expressed as mg /100 mg fresh tissue (mean \pm s.e. mean)*

	No of animals	Total phospholipids	Monoamino phospholipids	Lecithin	Sphingomyelin (total phospholipid less monoamino phospholipid)	Kephalin (monoamino phospholipid less lecithin)
Guinea pig	8	4 82 \pm 0 28	3 79 \pm 0 13	—	0 94 \pm 0 09	—
Cat	8	4 57 \pm 0 29	3 69 \pm 0 23	1 18 \pm 0 08	0 93 \pm 0 10	2 15 \pm 0 35
Rabbit	4	5 40 \pm 0 18	4 32 \pm 0 16	1 39 \pm 0 08	1 08 \pm 0 10	2 92 \pm 0 10

Table 3 *Lipids in grey matter and white matter of normal brain, expressed as mg /100 mg fresh tissue*

	Cerebroside	Free cholesterol	Total cholesterol	Ester cholesterol	Total phospholipid	Total 'essential lipids'
Cat 1 Grey matter	1 10	1 24	1 29	0 05	4 54	6 93
White matter	4 83	4 42	4 42	0 00	8 18	17 43
Cat 2 Grey matter	1 86	1 19	1 20	0 01	4 03	7 09
White matter	5 37	4 64	4 64	0 00	7 73	17 74
Dog 1 Grey matter	1 49	1 29	1 36	0 07	4 20	7 05
White matter	6 79	5 39	5 39	0 00	8 95	21 13
Dog 2 Grey matter	1 54	1 29	1 30	0 01	4 19	7 03
White matter	7 42	4 86	4 86	0 00	7 99	20 27
Beaver Grey matter	0 68	1 16	1 18	0 02	3 70	5 56
White matter	4 85	4 16	4 16	0 00	7 22	16 23
Human 1 Grey matter	1 20	1 00	1 00	0 00	3 12	5 32
White matter	4 61	3 69	4 00	0 31	6 24	14 85
Human 2 Grey matter	0 63	0 96	0 97	0 01	3 48	5 08
White matter	4 14	3 83	3 84	0 01	6 80	14 78

Table 4 *Phospholipids in grey matter and white matter of normal brain, expressed as mg /100 mg fresh tissue*

	Total phospholipids	Monoamino phospholipids	Lecithin	Sphingomyelin (total phospholipid less monoamino phospholipids)	Kephalin (monoamino phospholipids less lecithin)
Cat 1 Grey matter	4 54	3 86	—	0 68	—
White matter	8 18	6 02	—	2 16	—
Cat 2 Grey matter	4 03	3 29	1 35	0 74	1 94
White matter	7 73	3 95	1 36	3 78	2 59
Dog 1 Grey matter	4 20	3 26	0 77	0 94	2 49
White matter	8 95	4 66	1 65	4 29	3 01
Dog 2 Grey matter	4 19	3 28	1 23	0 91	2 05
White matter	7 99	4 95	1 62	3 04	3 33
Beaver Grey matter	3 70	2 90	0 69	0 80	2 21
White matter	7 22	4 54	1 13	2 68	3 41
Human 1 Grey matter	3 12	2 82	0 61	0 30	2 21
White matter	6 24	3 64	0 90	2 60	2 74
Human 2 Grey matter	3 48	2 93	1 16	0 55	1 77
White matter	6 80	4 99	1 49	1 80	3 50

(Table 3) Each of the individual phospholipids (lecithin, sphingomyelin and kephalin) was present in a greater concentration in the white matter than in grey, but by far the greatest difference was in the sphingomyelin fraction (Table 4)

In Table 5 the various lipid fractions have been expressed as a percentage of the 'essential lipid', i.e. as a percentage of the sum of the cerebroside, total cholesterol and total phospholipid. On this basis it

DISCUSSION

The methods used allow triplicate estimations of all the substances measured to be done on samples of tissue as small as 500 mg. If single determinations only are done, samples weighing as little as 100 mg may be used.

The extraction procedure and the published methods for the estimation of both free and total

Table 5 *Distribution of lipids in normal brain, expressed as percentage of 'essential lipids'*

		Cere broside	Total chole sterol	Total phospho- lipid	Lecithin	Sphingo myelin	Kephalin
Cat 1	Grey matter	15.8	18.6	65.6	—	9.8	—
	White matter	27.7	25.3	47.0	—	22.4	—
Cat 2	Grey matter	26.3	16.9	56.8	18.8	10.5	27.5
	White matter	30.3	26.1	43.6	7.6	21.4	14.6
Dog 1	Grey matter	21.4	19.2	59.4	10.7	13.6	35.1
	White matter	32.1	25.4	42.5	7.7	20.2	14.6
Dog 2	Grey matter	21.9	18.4	59.7	17.4	13.1	29.2
	White matter	36.6	24.0	39.4	8.0	15.0	16.4
Beaver	Grey matter	12.3	21.4	66.3	12.4	14.3	39.6
	White matter	29.9	25.8	44.3	6.9	16.6	20.8
Human 1	Grey matter	22.6	18.8	58.6	11.4	5.7	41.5
	White matter	31.1	26.9	42.0	6.0	17.5	18.5
Human 2	Grey matter	12.4	19.1	68.5	22.7	10.8	35.0
	White matter	28.0	26.0	46.0	10.1	12.2	23.7

is seen that, compared with grey matter, white matter contained relatively more cerebroside and cholesterol and less phospholipid. Of the individual phospholipids, however, white matter contained relatively more sphingomyelin and less lecithin and kephalin, thus, even although the concentration of total phospholipid in white matter was less on an 'essential lipid' basis, the concentration of sphingomyelin was greater. It follows, therefore, that the percentage of sphingomyelin in the total phospholipid fraction must be greater in white matter than in grey. This was observed to be so (Table 6)

Table 6 *Distribution of phospholipids in normal brain, expressed as percentage of total phospholipids*

		Lecithin	Sphingo myelin	Kephalin
Cat 1	Grey matter	—	14.9	—
	White matter	—	47.6	—
Cat 2	Grey matter	33.4	18.2	48.4
	White matter	18.4	48.0	33.6
Dog 1	Grey matter	18.3	22.4	59.3
	White matter	18.6	47.4	34.0
Dog 2	Grey matter	29.2	21.8	49.0
	White matter	20.3	38.1	41.6
Beaver	Grey matter	18.6	21.6	59.8
	White matter	15.7	37.1	47.2
Human 1	Grey matter	19.5	9.7	70.8
	White matter	14.3	41.7	44.0
Human 2	Grey matter	33.2	15.8	51.0
	White matter	21.9	26.6	51.5

cholesterol and of cerebroside proved satisfactory. At first sphingomyelin was measured by the reneckate method developed by Thannhauser and his colleagues (Thannhauser & Setz, 1936*a, b*, Thannhauser & Benotti, 1938). This method, combined with the estimation of the choline containing phospholipids, was used for determining the phospholipid distribution in tissues as described by Thannhauser, Benotti & Reinsteim (1939), and adapted for small samples of tissue by Erickson, Avrin, Teague & Williams (1940). The reneckate method proved unsatisfactory in our hands, however, and it is interesting to note that it has also been criticized by Hack (1946), Schmidt *et al.* (1946) and Thannhauser, Benotti & Boncoddo (1946).

There are few reports in the literature on the distribution of the lipids in the brain of the guinea pig, rabbit and cat, and, as far as we know, none on that of the beaver. The figures presented here are comparable to those reported for dog brain by Erickson *et al.* (1940), for ox brain by Kaucher, Galbraith, Button & Williams (1943) and for rat brain by Williams, Galbraith, Kaucher, Moyer, Allen & Macy (1945).

The finding that there was a greater concentration of cerebroside, cholesterol and sphingomyelin in white matter than in grey matter is in agreement with the work of Randall (1938) and Yasuda (1937), who found that there was a greater concentration of cholesterol and phospholipid in white matter than

in grey The greater concentration of cholesterol in white matter has also been observed by Petrowsky (1873), Thudichum (1901) and Kirschbaum & Linnert (1912), and the greater concentration of cerebroside by Petrowsky (1873) and Smith & Mair (1912-13)

In the present study it was shown that, on a wet weight basis, there was a greater concentration of all three phospholipids—lecithin, sphingomyelin, and kephalin—in the white matter than in the grey, but that the increase was chiefly in the sphingomyelin fraction This is in confirmation of the observation of Schmidt *et al* (1946), who found that the concentration, on a wet weight basis, of sphingo myelin in the white matter of ox brain was much greater than that in the grey matter

The figures reported for the relative concentrations of lecithin, sphingomyelin and kephalin for grey matter are similar to those obtained for whole brain by other workers using the method of Thannhauser, Benotti & Reinstein (1939) For instance, Thannhauser, Benotti, Walcott & Reinstein (1939) for human brain, Erickson *et al* (1940) for dog brain, Kaucher *et al* (1943) for ox brain, and Williams *et al* (1945) for rat brain found that the concentration of sphingomyelin was roughly the same as that of lecithin, and that the concentration of kephalin was much greater In white matter the concentration of sphingomyelin relative to that of lecithin was found to be much greater than in grey, whereas the concentration of kephalin relative to that of lecithin was not greatly different

White matter is thus seen to be characterized by a high concentration of 'essential lipid', there being relatively more cerebroside, cholesterol and sphingo

myelin than is present in grey matter, with little difference in the case of lecithin or kephalin This was found to be true for all the species studied, the cat, dog, beaver and man, and it is tempting to speculate that it is the cerebroside, cholesterol and sphingomyelin, rather than lecithin and kephalin, that form the basis of 'myelin'

SUMMARY

1 The concentration of cerebroside, free cholesterol, total cholesterol, total phospholipid, lecithin, sphingomyelin and kephalin has been determined in the whole brains of a series of guinea pigs, cats and rabbits

2 Similar studies have been made on both the white matter and grey matter from brains of the cat, dog, beaver and man

3 On a wet weight basis, there was a greater concentration of cerebroside, total cholesterol, total phospholipid, lecithin, sphingomyelin and kephalin in white matter than in grey matter

4 Referred to total 'essential lipid' the concentration of cerebroside and total cholesterol was greater in white matter than in grey, while the concentration of total phospholipid was less However, the concentration of sphingomyelin was greater in white matter, this being offset by a decrease in the concentration of both lecithin and kephalin

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Lipids of Peripheral Nerve

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In order to study experimentally the changes which occur in the distribution of the lipids in a peripheral nerve when it undergoes degeneration, methods had to be developed which demanded only a small sample of tissue. In this paper the application of the methods at present in use in this laboratory to normal peripheral nerve is described.

METHODS

Analyses were made on the sciatic nerves of 3 rabbits, 4 cats, 3 dogs and a beaver, and on the sciatic nerves or femoral nerves of a number of humans. The nerve was dissected out as soon as possible after death and the lipids were extracted and estimated by the methods described previously (Johnson, McNabb & Rossiter, 1948). The concentration of free cholesterol, total cholesterol, cerebroside, total phospholipid, monoaminophospholipid and lecithin was measured directly, and, from these figures, the values for ester cholesterol, sphingomyelin and kephalin were calculated.

RESULTS

The concentration of 'essential lipid', i.e. cerebroside, cholesterol and phospholipid, for each nerve studied is given in Table 1, and the concentration of lecithin, sphingomyelin and kephalin, the individual phospholipids which go to make up the total phospho-

lipid fraction, in Table 2. The figures in each of these tables are in terms of mg/100 mg fresh tissue. In Table 3, each of the lipid fractions is given as a percentage of the 'essential lipid', while in Table 4 each of the individual phospholipids—lecithin, sphingomyelin and kephalin—is given as a percentage of the total phospholipid.

DISCUSSION

The classical work on the distribution of the lipids in peripheral nerve is that of Falk (1908), who found the following relative concentrations of lipid in medullated nerve: cholesterol, 25.0, kephalin, 12.4, lecithin, 2.9, and cerebroside, 18.2. In the present investigation, in which more refined methods have been used for determining the distribution of the lipids, the results are considerably different.

Randall (1938), in a study of human nerve, found that the concentration of phospholipid was 4.5, cholesterol 1.5 and cerebroside 1.6%, referred to wet weight. In general our results for human nerve substantiate those of Randall, but the latter did not attempt to distinguish between the various phospholipid fractions. In most of the human nerves studied, the concentration of the various lipids was found to be slightly less than that reported by

Table 1 *Lipids in peripheral nerve (expressed as mg/100 mg fresh tissue)*

	Cere brosides	Free chole sterol	Total chole sterol	Ester chole sterol	Total phospho lipid	'Essential lipid'
Rabbit 1 (S)	2.94	2.68	2.70	0.02	5.21	10.85
Rabbit 2 (S)	2.61	2.67	2.80	0.13	5.79	11.20
Rabbit 3 (S)	3.23	3.65	3.69	0.04	6.81	13.73
Cat 1 (S)	2.88	3.19	3.23	0.04	6.21	12.32
Cat 2 (S)	1.48	3.56	3.64	0.08	5.88	11.00
Cat 3 (S)	2.82	3.74	3.75	0.01	7.27	13.84
Cat 4 (S)	1.99	2.87	2.89	0.02	6.28	11.16
Dog 1 (S)	2.88	3.07	3.10	0.03	5.39	11.37
Dog 2 (S)	4.15	2.78	2.78	0.00	5.14	12.07
Dog 3 (S)	1.97	2.43	2.43	0.00	5.62	10.02
Beaver 1 (S)	1.34	3.15	3.15	0.00	5.85	10.34
Patient 1 (S)	2.54	1.51	1.65	0.14	3.81	8.00
Patient 2 (F)	0.94	1.37	1.37	0.00	2.22	4.53
Patient 3 (S)	1.09	1.25	1.29	0.04	3.09	5.47
Patient 3 (F)	1.35	1.44	1.44	0.00	2.97	5.76
Patient 4 (S)	1.30	1.16	1.17	0.01	2.40	4.87
Patient 4 (F)	1.33	1.48	1.53	0.05	3.32	6.18

S=sciatic nerve, F=femoral nerve

Table 2 *Phospholipids in peripheral nerve (expressed as mg/100 mg fresh tissue)*

	Total phospholipid	Monoamino phospholipid	Lecithin	Sphingomyelin (total phospholipid less monoamino phospholipid)	Kephalin (monoamino phospholipid less lecithin)
Rabbit 1 (S)	5.21	2.74	0.67	2.47	2.07
Rabbit 2 (S)	5.79	3.54	0.66	2.25	2.88
Rabbit 3 (S)	6.81	2.94	1.10	3.87	1.84
Cat 1 (S)	6.21	3.22	0.72	2.99	2.50
Cat 2 (S)	5.88	3.25	0.72	2.63	2.53
Cat 3 (S)	7.27	2.76	1.01	4.51	1.75
Cat 4 (S)	6.28	3.13	0.96	3.15	2.17
Dog 1 (S)	5.39	2.09	1.03	3.30	1.06
Dog 2 (S)	5.14	2.23	1.01	2.91	1.22
Dog 3 (S)	5.62	2.38	0.72	3.24	1.66
Beaver 1 (S)	5.85	2.68	0.72	3.17	1.96
Patient 1 (S)	3.81	1.49	0.64	2.32	0.85
Patient 2 (F)	2.22	0.89	0.31	1.33	0.58
Patient 3 (S)	3.09	1.23	0.35	1.86	0.88
Patient 3 (F)	2.97	1.28	0.43	1.69	0.85
Patient 4 (S)	2.40	0.78	0.35	1.64	0.41
Patient 4 (F)	3.32	1.61	0.46	1.71	1.15

S=sciatic nerve, F=femoral nerve

Table 3 *Distribution of lipids in peripheral nerve (expressed as percentage of 'essential lipid')*

	Cere broside	Total whole sterol	Total phospho lipid	Lecithin	Sphingo myelin	Kephalin
Rabbit 1 (S)	27.1	24.9	48.0	6.2	22.8	19.0
Rabbit 2 (S)	23.3	25.0	51.7	6.0	20.3	25.4
Rabbit 3 (S)	23.6	26.8	49.6	8.2	27.9	13.5
Cat 1 (S)	23.4	26.2	50.4	5.8	24.1	20.5
Cat 2 (S)	13.4	33.1	53.5	6.6	23.9	23.0
Cat 3 (S)	20.6	26.5	52.9	7.4	32.8	12.7
Cat 4 (S)	17.8	25.9	56.3	8.5	28.4	19.4
Dog 1 (S)	25.3	27.3	47.4	9.1	29.0	9.3
Dog 2 (S)	34.4	23.0	42.6	8.4	24.1	10.1
Dog 3 (S)	19.7	24.2	56.1	7.2	32.4	16.5
Beaver 1 (S)	13.0	30.4	56.6	6.9	30.8	18.9
Patient 1 (S)	31.7	20.7	47.6	7.8	29.1	10.7
Patient 2 (F)	20.9	30.2	48.9	6.7	29.4	12.8
Patient 3 (S)	19.7	24.3	56.0	5.9	33.9	16.2
Patient 3 (F)	23.3	25.1	51.6	7.5	29.4	14.7
Patient 4 (S)	26.6	24.1	49.3	7.1	33.8	8.4
Patient 4 (F)	21.5	24.8	53.7	7.4	27.7	18.6

S=sciatic nerve, F=femoral nerve

Randall However, our material came from older patients, the ages of the four patients studied being 54, 68, 74 and 45 years. Randall has reported that, in diabetic and arteriosclerotic gangrene, there is a decrease in the concentration of each of the 'essential lipid' constituents of nerve, an observation confirmed by us for other degenerative conditions.

Another point to consider is that the material studied came from the proximal end of the nerve, where the nerve is large and the incorporated fibrous connective tissue much greater. This, we feel, explains why, on a wet weight basis, the concentration of 'essential lipid' in human nerve was found to be less than that of the rabbit, cat, dog and beaver, where the nerves were smaller and connective tissue less.

Despite the fact that the concentration of each of the lipid fractions was found to be less in human nerve, the distributions of the three fractions which make up the 'essential lipids', and of the individual phospholipids, were very similar in the human and animal nerves.

There are few data in the literature for the distribution of the lipids in the peripheral nerves of the rabbit, cat, dog and beaver. Schmidt, Benotti, Hershman & Thannhauser (1946) estimated total phospholipid and monoaminophospholipid concentration in three cat nerves. They found 7.5, 8.6 and 8.3% phospholipid, of which 50, 44.5 and 44.5% was sphingomyelin, these figures are of the same order as ours. They also found that sphingomyelin formed only 20% of the total phospholipid in rat

nerve, a value lower than that for any of the species investigated in the present study

Table 4 *Distribution of phospholipids in peripheral nerve (expressed as percentage of total phospholipid)*

	Lecithin (%)	Sphingo myelin (%)	Kephalin (%)
Rabbit 1 (S)	12.9	47.4	39.7
Rabbit 2 (S)	11.4	38.9	49.7
Rabbit 3 (S)	16.2	56.8	27.0
Cat 1 (S)	11.6	48.2	40.2
Cat 2 (S)	12.2	44.8	43.0
Cat 3 (S)	14.0	62.0	24.0
Cat 4 (S)	15.2	50.3	34.5
Dog 1 (S)	19.1	61.2	19.7
Dog 2 (S)	19.6	56.6	23.8
Dog 3 (S)	12.8	57.7	29.5
Beaver 1 (S)	12.3	54.4	33.3
Patient 1 (S)	16.5	61.1	22.4
Patient 2 (F)	13.6	60.2	26.2
Patient 3 (S)	11.3	60.2	28.5
Patient 3 (F)	14.5	57.0	28.5
Patient 4 (S)	14.5	68.4	17.1
Patient 4 (F)	13.8	51.5	34.7

S=sciatie nerve, F=femoral nerve

The relative distribution of cerebroside, cholesterol and phospholipid was roughly 1 : 1 : 2 in peripheral nerves of all the species studied. This is also roughly the distribution of the 'essential lipids' in brain tissue of the same species (Johnson *et al.* 1948). When one examines the distribution of the various constituents of the phospholipid fraction, however, a difference between peripheral nerve and brain tissue becomes apparent. In all the species studied 50–70% of the phospholipid of peripheral nerve is sphingomyelin, whereas the corresponding figure for whole brain tissue is 20%. The figure is 40% for

brain white matter, however, and in general the distribution of all the lipid constituents of peripheral nerve bears a much greater resemblance to the lipid distribution in the white matter of the brain than to either that of the grey matter or that of the brain as a whole. This is particularly apparent when the lipids are presented as percentages of total 'essential lipid', and it is, perhaps, hardly surprising when one considers that a high percentage of the lipids in both the white matter of the brain and in peripheral nerve go to make up the 'myelin' sheath. The chief difference between the lipid distribution in peripheral nerve and that in white matter of the brain is that, in peripheral nerve the percentage of sphingomyelin is somewhat greater, while the percentage of kephalin and cerebroside is somewhat less.

SUMMARY

1 The cerebroside, free cholesterol, total cholesterol, total phospholipid, lecithin, sphingomyelin and kephalin concentrations in peripheral nerves from the rabbit, cat, dog, beaver and man have been determined.

2 The distribution of the 'essential lipids' (cerebrosides, total cholesterol and total phospholipid) and of the individual phospholipids more closely resembles the distribution in the white matter of the brain than that in either the grey matter or whole brain. In peripheral nerve, however, there is relatively more sphingomyelin and less kephalin and cerebroside than in brain white matter.

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Effect of Adrenalectomy on the Arginase Levels of Liver, Mammary Gland and Kidney in Lactating Rats Studied by the Paired Feeding Technique

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The liver arginase in the rat decreases following adrenalectomy (Fraenkel Conrat, Simpson & Evans, 1943, Folley & Greenbaum, 1946, Kochakian & Vail, 1947), and in lactating rats the mammary gland arginase behaves similarly (Folley & Greenbaum, 1946). It is possible that the decline in lactation caused by adrenalectomy (see, e.g., Cowie & Folley, 1947a) is connected with the decrease in mammary gland arginase, for there is evidence (Folley & Greenbaum, 1947a) that this enzyme system plays an important role in the metabolism of the lactating mammary gland.

Since adrenalectomy is often followed by a marked reduction in food intake (see Ingle, 1944), it is important to find out whether the depressed tissue arginase levels characteristic of adrenalectomized animals are a primary consequence of the loss of the adrenals or merely a secondary consequence of the post operative anorexia. Particularly is this so for lactating rats, in which the food intake rises rapidly and steadily over the first 17 days of the lactation period to a value some three times that of pregnancy, so that even though the effect of adrenalectomy during lactation is to prevent this rise rather than to cause an actual fall, the disparity in food intake between adrenalectomized and intact rats soon becomes very marked (Cowie, Folley, French & Greenbaum, 1948).

An investigation of this question by the paired feeding method is described below. The arginase levels of the kidney were studied in addition to those of mammary gland and liver, since Kochakian & Vail (1947) have reported that the kidney arginase, like that of liver and mammary gland, is decreased after adrenalectomy.

EXPERIMENTAL

Animals Hooded Norway rats were used, approx. 5-6 months old and undergoing their first lactation. The diet was the latest version of the stock diet used in this colony in which 10% of the parts of whole wheat have been replaced by wheat germ (see Cowie & Folley, 1948). The technique of paired feeding, including certain small obligatory dietary modifications, was as used in a previous study by Cowie & Folley (1948). Litters were reduced to 8 at birth and the mothers adrenalectomized or sham operated on the 4th day, with autopsy on the 17th day.

Tissue arginase determination Enzyme assays were made on tissue homogenates made as described previously (Folley

& Greenbaum, 1947a), liver, 'abdominal'* mammary glands and kidney being dispersed for 5 min into 9, 19 and 29 parts respectively of normal saline. The homogenates were fully activated with Mn^{++} after preparation (see Folley & Greenbaum, 1948a) so that the results give the presumed levels of enzyme protein in the tissues. It was shown previously (Folley & Greenbaum, 1948a) that, in view of difficulties at present unsurmountable in the way of determining the degree of activation of the 'native' arginase system, the 'potential' arginase, as given by the activity of Mn^{++} activated homogenates, is probably the most biologically significant determination that can be made, particularly since such evidence as we have indicates that the enzyme is almost fully activated in the tissues of our rats.

The determinations were made by the method of Folley & Greenbaum (1948a), the reaction being carried out at 37° and pH 9.45 in presence of 0.227 M substrate. The reaction times were as follows: liver, 5 min; mammary gland, 5 or 10 min; kidney, 10 min. The results are expressed in arbitrary arginase units as defined by Folley & Greenbaum (1948a). As in a previous study (Folley & Greenbaum, 1947a) the mammary gland results have been expressed on a 'milk free' tissue basis, the lactose content of milk, secreted by adrenalectomized and pair fed sham operated rats, being assumed to be the same as for normal lactating rats. Since this assumption may not be quite true (see discussion by Folley & Greenbaum, 1948b) the results for the mammas of the two former groups may be subject to a slight error.

RESULTS

Table 1 gives data relating to the body weights of the rats, organ weights, and milk content of the mammary tissues, etc. The tissue arginase levels are given in Table 2. Of the mammary glands only the 'abdominal' ones were considered suitable for this work, and thus it was impossible to calculate the total mammary gland arginase activity per 100 g rat, instead, values for the total 'abdominal' mammary gland arginase per 100 g rat are given.

Table 2 shows that the arginase activities of the tissues of sham operated rats pair fed with adrenalectomized rats were not significantly different from those of controls fed *ad lib* (see probabilities in Table 3). The total liver and 'abdominal' mammary-gland arginase values were, however, significantly diminished in the pair-fed controls due to a marked reduction in the size of these organs (Table 1). The total arginase of the kidney, which decreased very

* I.e. the pair of abdominal and the two pairs of inguinal glands.

post adrenalectomy changes noted in the arginase levels of the mammary gland and kidney, however, it would seem reasonable to conclude that anorexia is not the primary cause of the decrease in the liver arginase following adrenalectomy

The use of an arginase assay method kinetically more sound (see discussion by Folley & Greenbaum, 1948*a*) than that used previously for the purpose (Folley & Greenbaum, 1946), together with the introduction of a means of making at least an approximate correction of the results for the milk retained in the tissue, has enabled us to make a re evaluation of the arginase activity of the mammary gland in relation to other arginase containing tissues in the lactating rat, which is considerably more favourable to the mammary gland than our previous estimate. Since we have now found that, in the intact lactating rat, the mammary gland exhibits about three times the arginase concentration of the kidney, our previous conclusion (Folley & Greenbaum, 1946) that the mammary gland is the second most abundant source of mammalian arginase is amply confirmed. These calculations of course refer to the total 'activatable' enzyme (presumably the total enzyme protein), but it is not anticipated that the results would be much upset if it were possible to determine the concentration of the active Mn^{++} protein (see Folley & Greenbaum, 1948*a*) in the tissues. Our liver/kidney ratio is lower than the ratio reported by Kochakian (1944) for the mouse. Assuming, by analogy with the constancy of the concentration of alkaline phosphatase in the rat kidney during pregnancy, lactation and involution (Folley & Greenbaum, 1947*a*), that the kidney arginase does not increase in lactation, this discrepancy could be accounted for, at least partly, by the increased liver arginase content during lactation (Folley & Greenbaum, 1947*a*).

SUMMARY

1 Liver, kidney and mammary-gland arginase levels of rats, adrenalectomized on the 4th day of lactation and autopsied on the 17th day, were significantly lower than those of pair-fed sham operated lactating rats. Arginase per g moist tissue was reduced most in the mammary gland (to one seventh) and least in the kidney (to one half).

2 The tissue arginase levels of sham operated lactating rats pair fed with adrenalectomized rats were not significantly lower than those of sham operated rats fed *ad lib*, save in the case of total arginase in the liver and in the 'abdominal' mammary glands, the reduction in which was in each case due to a decrease in the size of the gland or glands concerned.

3 It is concluded that the results indicate a closer relationship between adrenal cortical hormones and the arginase of the mammary gland and kidney than would be the case if the decrease in the enzyme concentration were due primarily to post operative anorexia. Reasons are advanced for the belief that the diminution of liver arginase following adrenalectomy is also not primarily due to anorexia.

4 A re evaluation of the relative arginase activity of the mammary gland, in relation to that of other tissues in the intact lactating rat, indicates that liver contains nine times the amount in mammary tissue and twenty eight times the amount in kidney. These ratios are much the same in intact lactating rats on a reduced food intake, but in adrenalectomized rats the activity of the mammary gland relative to the liver falls slightly below that of the kidney.

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Vitamin C in the Walnut (*Juglans regia*)

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The remarkably high concentration of vitamin C in unripe walnuts (*Juglans regia*) appears to have been first reported by Gerghelezhru (1937), and has been confirmed by Pyke, Melville & Sarson (1942) who also examined a number of allied species and genera. Melville, Wokes & Organ (1943) showed that unripe walnuts and nuts from allied species may also contain considerable amounts of apparent vitamin C, the provisional term suggested by Wokes, Organ, Duncan & Jacoby (1943) to describe substances occurring in certain foods, and, although not possessing the antiscorbutic potency of vitamin C, not distinguished from it by the indophenol dye titration as usually carried out. Differentiation between true and apparent vitamin C in walnuts can, however, be effected by modifications of Lugg's (1942) formaldehyde method (Mapson, 1943, Wokes, Organ & Jacoby, 1943).

During the last four years we have studied the distribution of true and apparent vitamin C in different tissues of the walnut at intervals throughout the season, estimating them in the minute amounts of material permitted by the high concentration of the vitamin. Our data provide information on the possible functions and phytosynthesis of vitamin C which may be applicable to other plant material containing this vitamin.

METHODS

Collection of material

The majority of the samples were taken from a typical tree of *J. regia* growing at Kew, and the results were checked on a second tree at Kew and others at Berkhamsted and King's Langley. The material was at once put into air tight boxes and transported as quickly as possible to the laboratory, where it was either examined immediately or stored in the refrigerator. Tests showed that there was no significant loss of vitamin C during transport, and very little change in moisture content except in leaves, where as much as 10% might be lost between collection and dissection. In view, however, of the variable moisture content of the leaves as collected at different times of day, and also of probable changes in weight through varying metabolic activity during the day, we adopted Chibnall's (1923) procedure and expressed our results on the basis of fresh weight. Since our findings were largely comparative we do not think this procedure led to serious errors as far as leaves were concerned. With fruits, stems, buds, catkins and other parts the moisture losses were insignificant.

Preparation of extracts

Nuts The walnut is a drupe like fruit in which the outer green husk is derived from two whorls of fused bracts. In this paper the epidermis and a few subjacent cell layers are referred to as epicarp, and the bulk of the green husk belonging to the inner bracts as mesocarp. The white inner portion, which eventually becomes woody, corresponds with endocarp and consists of two carpels. The testa or seed coat is well developed at an early stage. The endosperm remains fluid until the fruit is almost full size, the shell beginning to harden before the endosperm is replaced by the embryo. An attempt was made to separate these tissues from one another and to follow the seasonal changes of vitamin content in them. Shallow cuts were made along the line of union of the two carpels, which were forced apart and the undamaged kernel obtained free from adherent endocarp. The epicarp was then removed in thin slices, followed by the mesocarp, and finally the endocarp was cut in small pieces. Separation of mesocarp from endocarp was ensured by rejecting adjacent parts of these tissues. If a separate sample was required of the liquid contents of the kernel in young nuts, this was obtained by piercing the unbroken testa with a fine pointed test pipette and with drawing the liquid by suction. Any of the liquid coming in contact with endocarp was removed by filter paper. When a sample of the larger whole nut was taken, the first cut along the carpellary junction was followed by a second cut at right angles, each cut being taken right to the centre to include the kernel. The two diagonally opposite quarters were taken for duplicate samples of the whole nut, and the remaining two quarters used for samples of epicarp, mesocarp and endocarp.

Peduncle or fruit stalk With the object of studying gradients of vitamin C within the plant a rough separation of phloem from xylem and other tissues was effected. In the fruit stalk longitudinal slices of cortex were cut off until the circular zone of pericyclic fibres was reached, as determined by their paler colour. Continuing with longitudinal slices, the tissues from the pericyclic fibres to the cambium were included as phloem, and those inside the cambium as xylem and pith.

Stem A ring of bud scale scars marked the limit of the previous year's growth. Samples of cortex with phloem of the current year were obtained by peeling off the bark, which came away readily at the cambium. The remaining white cylinder consisted of xylem with pith. In older bark the phloem could be separated from the cortex by careful dissection.

Leaves The petiole was freed from its swollen base. The petiolar cortex was separated from the remaining tissues by removing longitudinal slices until the zone of pericyclic

fibres was reached. The green tissue of the leaflets, consisting mainly of mesophyll, was separated by cutting along the midrib and its main branches.

Buds and catkins The cone like male catkin buds were readily identified on reproductive shoots but female catkin buds are not differentiated in *J. regia*. Since the nuts always develop from terminal buds, the terminal and lateral buds of both vegetative and reproductive shoots were examined separately.

Roots These were separated into fine and coarse but were not dissected. In order to minimize losses through enzymic action all pieces cut off were placed immediately in tared weighing bottles containing HPO_3 . Stainless steel knives and safety razor blades were used.

Estimations

Dye titrations Visual estimations of 'total' vitamin C were made substantially as described by Harris & Olliver (1942) for true vitamin C, the end point being persistence of the pink colour for 20 sec, and care being taken with the timing when there was sufficient apparent vitamin C present to affect the rate of reaction with the dye. 'Apparent' vitamin C was estimated by the method of Wokes, Organ & Jacoby (1943). The methods of Mapson (1943) and of Snow & Zilva (1944) applied occasionally, when sufficient material was available, showed certain discrepancies because they do not make adequate allowance for the effect of the titration pH on the dye titration value, which has been shown to be very marked in walnut material (Wokes, 1946). 'True' vitamin C was usually determined indirectly by subtracting apparent from total vitamin C. It was occasionally estimated directly by the ascorbic acid oxidase method, based on the fact that the apparent vitamin C in walnuts acts much more slowly with the enzyme than does true vitamin C. When calculating the concentration of apparent vitamin C the latter was assumed to have the same dye titration value as ascorbic acid. Evidence will be given later in support of this assumption.

Potentiometric estimations were made by the method of Harris, Mapson & Wang (1942) for true vitamin C, with certain modifications advantageous when apparent vitamin C is present (Wokes, Organ & Jacoby, 1943).

Dehydroascorbic acid Estimations of this substance by the H_2S reduction method showed that it could only have been a small percentage of the total vitamin C content (e.g. leaves 3-4%, nuts 3-5%, endocarp 5%). Since sufficient material for accurate determination of these minute amounts was often lacking, such estimations were usually omitted.

pH measurements These were made with a Cambridge portable pH meter, also used in the potentiometric estimations.

RESULTS

Oxidizing enzymes and pH of tissues

Study of these enzymes by the technique previously applied to tomatoes (Wokes & Organ, 1943) showed that the tissues, when examined at pH 6.5, can be placed in the following decreasing order of oxidative activity—epicarp, mesocarp, endocarp, kernel. The natural pH of the endocarp is usually below 5, causing a further decrease in activity. On the other

hand, the natural pH of the epicarp seems always to be above 5 and may reach 7, causing an increase in oxidative activity. The pH of the mesocarp may be above 7 in very small nuts, but gradually falls during growth and may be below 5 in full grown nuts. Hence, when dissecting walnut fruits for vitamin C assay, particular care should be taken with the epicarp and mesocarp. If these tissues are disintegrated, oxidizing enzymes may destroy half the vitamin and apparent vitamin C within a few minutes. By avoiding prolonged exposure of cut surfaces to air, and by dividing the tissues into not too small pieces, which were dropped immediately into HPO_3 solution, the losses due to oxidizing enzymes are believed to be reduced to a small proportion of the total error. Green tissues outside the nuts, such as fruit stalk, stem, leaves and buds, appear usually to be neutral and to contain considerable amounts of the oxidizing enzymes, and similar precautions are necessary.

Whole fruits (nuts)

Table 1 summarizes our results on nearly 100 *J. regia* nuts, most of which came from the main tree at Kew. They show that the average concentration of vitamin C in very young nuts (0.5-1.0 g) was 500-1000 mg/100 g. As the nuts grew, this concentration at first rapidly increased, being doubled within a fortnight, and reached a peak of about 2000 mg/100 g when the nuts weighed 2-3 g. In the Kew samples this peak occurred in 1944 towards the end of June and in 1945 about a fortnight earlier. In 1946 maturation was later than in 1945, the average concentration on 18 June being less than on 30 May in 1945. After the peak concentration was reached the nuts continued to grow rapidly, but the vitamin concentration gradually diminished, being only a half to two thirds of the peak concentration when the nuts were fully grown. Hence the total amount of vitamin C in a given nut increased more and more slowly during the later stages of maturation, and usually reached its maximum before the nuts were fully grown. It then remained fairly constant for several weeks, until formation of the woody shell began, when it fell rapidly. These findings on the main Kew samples were confirmed by results on nuts taken from other trees in 1943-6.

Different tissues in the nuts

These were obtained from nuts belonging to the same samples that provided the data on whole nuts summarized in Table 1. In the smallest nuts it was not possible to separate epicarp completely from mesocarp. The figures for these two tissues in the first two or three samples are, therefore, less accurate than those for later samples in the season (Table 2). In larger nuts, where differentiation between epicarp

Table 1 *Seasonal changes in vitamin C concentration in walnuts*

(Nuts in samples 1, 2, 25 and 45 were too small to be examined individually and therefore were pooled. Weights of nuts taken immediately before examination do not differ by more than 1 or 2% from weights as taken from tree.)

Sample no	Date gathered	No of nuts	Average wt of nuts (g)	Vitamin C (mg/100 g)				Vitamin C (mg/nut, mean)
				Individual nuts			Mean	
			(a) From main tree					
1	7 June 1944	6	1		Pooled		—	5
2	13 "	10	1		Pooled		—	10
3	21 "	3	3	2080	1975	1932	1991	60
4	26 "	4	3.6	2080	1940	1920	2090	72
5	4 July	3	10	2085	2045	1915		201
6	12 "	4	20.3	1450	1615	1675	1690	327
7	18 "	4	17.5	1495	1575	1390	1530	262
8	25 "	4	22.6	1563	1335	1405	1563	332
9	1 Aug	3	24.9	1155	1180	1295		301*
10	9 "	2	32.1	1115	835			313*
11	22 "	2	21.7	1150	1280			264
25	17 May 1945	8	0.3		Pooled		928	3
26	30 "	3	0.5	1273	999	931		5
27	6 June	2	1.75	1848	1789			32
28	12 "	3	2.8	1836	2193	1983		56
29	19 "	3	4.1	2074	1799	1677		62
30	26 "	3	14.0	1490	1442	1468		205
31	10 July	3	25.2	1007	1357	1042		286
32	23 "	3	29.7	999	1022	972		297
33	8 Aug	3	32.0	905	1256	882		329
45	18 June 1946	13	0.3		Pooled		699	2
			(b) From other trees					
242	29 June 1944	3	10.6	2035	2025	2060	2036	216
243	25 June 1945	6	18.5	2037	1770	1732		
				1938	1934	1683	1849	342

* Excluding woody portion

Table 2 *Seasonal distribution of vitamin C in different tissues of walnut fruits*

(The number of nuts in each sample and date of collection are given in Table 1. The following figures are the sample averages.)

Sample no	Vitamin C (mg/100 g) in					
	Fruit stalk	Epicarp	Mesocarp	Endocarp	Testa	Endosperm
1	259	25	—	951	—	—
2	—	360	—	1644	—	—
3	—	791	—	3040	—	—
4	270	940	815	2980	—	—
5	235	985	1000	2855	—	10
6	—	1325	740	2255	65	9
7	—	1000	660	2155	75	20
8	—	1075	740	2280	66	21
9	60	980	750	2120*	65	13
10	—	963	745	2220*	71	22
11	220	1160	1170	3150*	33	21
26	273	342	1290	1532	—	—
27	330	782	1202	2641	—	—
28	189	918	986	2655	—	—
29	272	992	931	3083	—	—
30	246	1002	916	2406	—	—
31	134	921	784	1991	—	—
32	267	913	706	1800	—	—
33	316	408	547	1728	—	53†
34	205	808	867	—	—	31†
241	—	1170	880	3002	—	—
242	—	1195	1085	3140	—	—
243	—	1410	1203	2678	—	140†

* These results are averages on endocarp excluding wood. If wood is included, average results for samples 9 and 10 are 1790 and 1410 respectively.

† These results were obtained on whole kernel, including both testa and endosperm.

and mesocarp was more complete, the concentration of vitamin C in the epicarp was sometimes but not always higher than in the mesocarp. The peak concentration was generally reached at a later date in the epicarp than in the mesocarp or endocarp. The concentration in the endocarp was always much higher than in other tissues, but fell rapidly when lignification of the shell began. It seemed possible that, during the dissection of the epicarp and mesocarp, a loss of vitamin C occurred which might explain why the concentration of this vitamin in these two tissues was so much lower than in the endocarp. We, therefore, compared the sum of the results on the different tissues in a number of dissected nuts with those on whole nuts from the same samples (Table 3). Allowing for the weights of the different tissues, the results on the dissected nuts were very similar to those on the corresponding whole nuts, ranging from 84 to 119 and averaging 99% of the latter in 15 nuts from five samples. We, therefore, felt satisfied that our findings were not being affected seriously by loss of vitamin C during dissection. The kernels always contained very little vitamin C, but the concentration in the testa was always higher than in the endosperm, when separation of these two tissues was effected. These findings on the main Kew samples in 1944 and 1945, confirmed by results on other samples in 1943-6, made it difficult to assume that the large amount of vitamin C in the endocarp had come entirely, or even mainly, from the epicarp and mesocarp where the concentration was always much lower. We, therefore, considered as another possibility translocation of the vitamin from the leaves

Table 3 *Loss of vitamin C during dissection of walnuts*

(a) Dissection of nut 31/3

	Vitamin C		
	Wt of tissue (g)	Concn (mg/100 g)	Content (mg)
Epicarp	2.23	921	20.5
Mesocarp	16.87	784	132.2
Endocarp	7.10	1991	141.4
Kernel	2.64	0.50	1.3
Total	28.84		295.4

Average vitamin C concentration = 1007 mg/100 g

(b) Vitamin C concentration (mg/100 g)

In whole nut 31/1	1357
In whole nut 31/2	1042
	Mean 1200
In dissected nut 31/3	1007
In dissected as percentage of whole nuts	84
Percentage assumed loss during dissection which scarcely exceeds sampling error	16

Leaves

In whole leaflets from the main Kew tree the average concentration of vitamin C was found to be highest in the earlier part of the season (May and June) and to fall from a peak of about 1000 mg/100 g to less than half this concentration as the season progressed (Table 4). These leaflets were on the same shoots as, and therefore adjoined, the nuts of the corresponding samples in Tables 1 and 2. Being nearest to the nuts, they formed the most likely

Table 4 *Vitamin C in walnut leaves and stems*

Sample no	Date gathered	Vitamin C (mg/100 g) in					
		Whole leaflets	Mesophyll	Vascular tissue	Rachis	Petiole	Main stem cortex and phloem
4	26 June 1944	856	—	—	—	—	—
9	1 Aug	440	—	—	—	60	—
11	22 "	—	700	240	—	120	—
25	17 May 1945	876	930	663	458	308	353
26	30 "	908	1039	557	391	170	153
27	6 June	838	970	530	447	264	192
28	12 "	1037	1137	758	383	339	305
29	19 "	755	758	477	293	151	64
30	26 "	698	869	287	239	90	102
31	10 July	717	878	483	275	176	112
32	23 "	553	668	260	202	104	635
33	8 Aug	530	715	231	171	105	239
34	11 Sept	503	520	150	171	40	708
35	2 Oct	319	—	—	165	46	335
36	8 "	741	631	340	322	255	217
37	15 "	388	308	179	38	60	275
38	22 "	371	278	70	124	81	272
43	15 Apr 1946	227	—	—	—	—	83
44	29 "	230	—	—	530	438	122
45	18 June	814	853	750	475	445	235
46	1 July	1048	1161	804	450	350	245

source of any vitamin C passing into the latter from the leaves. The weight of each leaflet increased rapidly during the first few weeks after unfolding of the bud, but had become fairly constant by the beginning of June. The amount of vitamin C in each leaflet reached a maximum of about 10 mg. in June or July and then fell gradually. The total amount of vitamin C in the leaves examined after this date was perhaps 5-10 % of the amount in the nuts borne on the corresponding shoots. Examination of different leaflets in a leaf showed no marked variation in vitamin C concentration, the lowest concentration being usually not more than 10 % below the highest concentration. In our dissection of leaflets differentiation of mesophyll from vascular tissue, though necessarily incomplete, was sufficiently efficient to reveal marked differences between the vitamin C concentrations in the two tissues, that in the mesophyll always being much higher. The peak concentration in the mesophyll coincided with that in the whole leaflet. To test for any loss of vitamin C during dissection of the leaflets we compared in a number of leaflets the sum of the results obtained on the dissected mesophyll and vascular tissue of the leaflet with the concentration of vitamin C found in the corresponding whole leaflet on the opposite side of the leaf. Allowing for the weights of the dissected tissues, there was good agreement between the dissected and whole leaflets throughout the period when nuts were on the trees, the weighted mean concentration in the dissected leaflets ranging from 87 to 110, and averaging 97 % of the concentration in the corresponding whole leaflets in 12 samples collected from May to September. In three samples of older leaves collected on 9, 15 and 22 October the percentages fell to 76, 71 and 59 respectively, probably because the oxidizing enzymes were liberated more readily from the senescent cells. However, for the period when nuts were on the tree we felt satisfied that our findings were not being seriously affected by loss of vitamin C during dissection.

When studying the possible translocation of vitamin C from the mesophyll of the leaf to other parts of the walnut tree we determined its concentration in the rachis and petiole through which it would have to pass *en route* to the stem. Data from 17 samples summarized in Table 4 show that there is always a fall in concentration in passing from the vascular tissue of the leaflets through the rachis to the petiole. Separation of the cortex and phloem of the petiole by dissection from the xylem and pith showed that the concentration of vitamin C in the outer tissues was three to four times that in the inner, whereas the proportions by weight were approximately equal. The concentration in the outer tissues was therefore, at least twice that in the rachis or petiole. This distribution suggests that the vitamin is translocated mainly, if not entirely, in the phloem.

It was not possible to separate the phloem completely from other tissues for a vitamin C determination, but an estimate of the amount of phloem present from transverse sections suggests that the concentration in the phloem is probably not less than three times that reported for the whole rachis and petiole in Table 4.

It should be stated that invariably our results on the mesophyll and vascular tissue of the leaf, the rachis, petiole and the cortex and phloem of the stem were obtained on samples taken from adjoining parts of the same shoot.

Stem

The outer tissues, cortex and phloem were usually separated from the xylem and pith. The concentration of vitamin C in the former was found to be three to four times that in the xylem and pith and the results from 18 samples are included in Table 4. These were all obtained from stems of the current year's growth (C Y G) to which the leaves analyzed were attached. In order, however, to obtain a more complete picture of the distribution of vitamin C in different parts of the stem, we extended our observations to other parts of the stem, including the previous or last year's growth (L Y G) and still older parts originating as far back as 1920. Stems were also examined in the winter when leaves were absent. The data on 34 samples, collected between August 1944 and October 1946, are summarized in Table 5. The wide variations from one sample to another are due probably to varying amounts of brittle cortex adhering to the phloem. Examination of the separated phloem in a number of samples low in vitamin C (e.g. nos. 21, 22, 26, 27) showed that about twice as much vitamin C was present in the phloem alone as in the combined tissues. The dissection of cortex from phloem was a tedious procedure needing 2 or 3 hr. to obtain a sufficient quantity for an assay, and time was not always available to prepare replicates from all the samples. However, our data suggest that the concentration of vitamin C in the phloem of stems of the current year is usually above 200 mg./100 g. and may reach a peak between 600 and 1000 mg./100 g. The concentration in the phloem of older stems is usually lower than in that of the current year.

Buds and catkins

The difference in vitamin C concentration between the lateral and terminal buds of vegetative shoots in any one sample was found to be smaller than that between either lateral or terminal buds of successive samples. During the autumn and winter when the buds were very small we therefore economized material by pooling lateral and terminal buds, the results being quoted as for vegetative buds in Table 5. There was likewise no significant difference

Table 5 *Seasonal distribution of vitamin C in buds, catkins and stem*

(Older stem sample corresponded to growth in previous years back to 1920 'Reproductive buds' were terminal buds on reproductive shoots, which might produce female flowers O Y G = current year's growth, L Y G = last year's growth)

Sample no	Date gathered	Vitamin C (mg/100 g) in					
		Stem (cortex and phloem)			Buds		Male catkins
		O Y G	L Y G	Older	Veg	Reprod	
12	29 Aug 1944	135	—	—	—	—	270
13	4 Sept	—	—	—	169	20	40
14	11 "	—	—	—	35	106	—
15	10 Oct	160	—	—	174	109	65
16	14 Nov	215	—	—	270	200	130
17	20 "	160	—	—	210	—	80
18	27 "	170	—	—	90	100	180
19	8 Jan 1945	499	147	—	163	—	460
20	21 "	222	177	178, 98	401	266	603
21	28 Feb	113	298	328	199	511	169
22	13 Mar	185	130	170	61	155	505
23	4 Apr	158	—	—	315	232	133
24	13 "	—	—	—	—	—	61
25	17 May	353	379	—	500	946*	—
26	30 "	153	188	—	617	1068*	—
27	6 June	192	163	112, 93	389	—	—
28	12 "	305	119	108	507	—	—
29	19 "	64	51	59	293	—	—
30	26 "	102	63	—	194	—	—
31	10 July	112	93	—	231	—	—
32	23 "	635	267	—	203	—	—
33	8 Aug	239	173	—	287	—	—
34	11 Sept	708	84	80, 53	99	—	357
35	2 Oct	335	260	378, 226 70, 141	63	54	873
36	9 "	217	240	175	197	—	1001
37	15 "	275	217	—	406	—	825
38	22 "	272	278	—	492	—	207
39	31 "	260	216	—	396	—	403
40	—	327	184	—	226	—	525
41	15 Jan 1946	202	160	—	150	—	220
B	21 Mar	240	—	—	—	—	364
42	2 Apr	120	70	—	236	214	115
43	15 "	83	59	—	159	—	100
44	29 "	122	40	—	—	—	64
45	18 June	235	199	—	—	—	—
46	1 July	245	354	—	1042	—	—
47	23 Sept	—	—	—	—	—	542
48	24 Oct	85	139	192	338	—	420

* Results obtained on female catkins

between the lateral and terminal buds of reproductive shoots during the autumn and winter, when the concentrations were similar to those in the buds from the vegetative shoots, i.e. 225 and 211 mg/100 g respectively. In the spring there were marked increases in vitamin C concentration in both types of bud shortly before they opened. After this the concentration fell to an average of about 200 mg/100 g in the unopened vegetative buds. In the autumn when reproductive buds were again available the vitamin C concentration in both kinds of buds had fallen to a still lower level.

Male catkin buds, on account of their shape, were much easier to distinguish than female reproductive buds. Data on them were obtained for 12 samples

gathered at regular intervals between 11 September 1945 and 29 April 1946 (Table 5), and show rather wide fluctuations in vitamin C concentration. However, the vitamin C content per catkin was found to be more uniform. From August until the end of the year the weight of the catkins remained fairly constant at 50–70 mg and the vitamin C content was usually less than 0.1 mg. Early in the following year the catkin buds grew rapidly until by March or April they weighed 0.5–1.0 g and their vitamin C content increased up to 1.0 mg. Soon afterwards the catkins shed their pollen and fell off. When catkin buds next appeared in the autumn, their content of vitamin C was at the initial low level of about 0.1 g.

DISCUSSION

When unripe walnuts were found to contain remarkably high concentrations of vitamin C, its origin and functions in the plant became of interest. In view of much evidence that in other fruits the vitamin C content is influenced by the degree of exposure to sunlight, it seemed likely that the source of the vitamin might be green tissues, such as the epicarp of the fruit or the leaf mesophyll where photosynthesis is most active. If the vitamin is synthesized in the epicarp, and translocated to the endocarp it would have to move against a steep concentration gradient. Such a movement could not take place by simple diffusion, but is not impossible where activities of living cells are involved (Stiles, 1936). In order to establish a gradient favourable to direct diffusion from epicarp to endocarp it would be necessary to show that the vitamin was confined to particular cells or regions of the epicarp which in some instances would have to contribute less than a quarter of the total weight of the tissue. Although no chemical evidence was obtained on this point, a segregation of morphologically similar cells into a tissue meshwork was observed in sections in which certain cells became brown on exposure, probably by the oxidation of juglone derivatives, while adjacent cells remained unstained for some time. Such a segregation of materials in the outer tissues of the fruit is rendered less unlikely by the existence of considerable differences in vitamin C concentration in the mesocarp and endocarp, the limiting cell layers of which must be extremely effective barriers to the reverse movement of the vitamin in order that the concentration may be maintained in the endocarp. This may have an evolutionary background in that the green husk of the walnut is not a true mesocarp, but arose by the fusion of several bracts with the true fruit represented by the endocarp and its contents. The presence of such boundary layers renders it probable that translocation from epicarp and mesocarp takes place through their vascular tissues to the base of the fruit and thence up into the endocarp.

As it was not possible to establish the existence within the fruit of a gradient of vitamin C favourable to the translocation and accumulation of the vitamin in the endocarp, other parts of the plant were considered. Concentrations of over 1000 mg/100 g were found in the leaf mesophyll in May and June, while the concentration in the midrib and lateral nerves was generally about 40% of that in the mesophyll. As the mesophyll contained a meshwork of small nerves it is probable that the actual concentration in the green assimilatory cells was well over 1000 mg/100 g throughout the period from May to August, but may have fallen below this level in September and October. There was always a clear gradient from the mesophyll to midrib and through the rachis as far

as the base of the petiole. Beyond this point vitamin C in course of translocation from the leaf would have to pass through a zone of stem with a higher concentration in the cortex and phloem, and then through a zone usually of a still higher concentration in the peduncle of the fruit, before it could reach the endocarp. The low concentration in the xylem and pith of the stem renders it unlikely that translocation takes place through the wood, and indicates the phloem as the only possible tissue for this function. The dissection of the fruit stalk into cortex, phloem and xylem plus pith enabled us to show that the highest concentration of vitamin C was in the cortex, where it was about twice that in the fruit stalk as a whole, probably on account of the photosynthetic activity of the green parts. The concentration in the phloem as dissected was about equal to that in the whole stalk. The possibility of greater concentrations in the sieve tubes and companion cells of the phloem was considered, since movement of the translocate may be confined to them. Estimates of the area of true phloem consisting of sieve tubes, companion cells and phloem parenchyma were made from sections of the rachis, petiole, stem and fruit peduncle. Assuming the bulk of the vitamin C to be concentrated in these cells, and applying a factor to the observed data the irregularities in the gradient from leaf to endocarp were somewhat reduced. Only if companion cells alone are considered, and the assumption made that the bulk of the vitamin present in the phloem is confined in these slender cells, would the anatomical evidence be favourable to a positive translocation gradient from the leaf mesophyll to the endocarp of the fruit. In the absence of a method for isolating the companion cells it was not possible to confirm this theory. Thus with the available data we are unable to demonstrate that the vitamin C in the endocarp comes mainly either from the epicarp or the mesophyll.

An alternative hypothesis, that vitamin C is translocated as a precursor or other form of compound which can yield ascorbic acid after it reaches the endocarp, will be considered in a later paper. In this connexion it is interesting to note that, in contrast with many other fruits, the pH of many walnut tissues, both from the fruit and from other parts of the plant, is slightly alkaline. Only in the endocarp is the usual acidity sufficient to improve the stability of vitamin C, whilst the concentration of ascorbic acid oxidase is much lower than in other walnut tissues. Thus any such precursor of vitamin C may be expected to differ from ascorbic acid in being resistant to the action of ascorbic acid oxidase and being stable in neutral solution (pH 6-8).

The function of vitamin C in plants is often stated to be concerned with growth. Its occurrence in higher concentration in young actively growing

shoots supports this hypothesis. Our data have shown that the vitamin C concentration in the vegetative buds increased markedly just before opening, the peak occurring in very young leaves. In the reproductive buds and catkins there was a similar increase just before rapid growth began. In the phloem of the stem, adjoining the cambium, there was a peak concentration at the time of active growth. Schopfer (1943) has suggested that vitamin C may also be connected with photosynthesis and the production of sugars. Our data support this suggestion in so far as high concentrations of the vitamin were found in the mesophyll and in other green tissues (e.g. cortex of green stems) where photosynthesis was most marked. However, photosynthesis cannot very well explain the function of the vitamin C in the endocarp, where the highest concentrations occur. Since these concentrations fall rapidly when the woody shell is developing, and since in a given nut the concentration of vitamin C in the woody part is much lower than in the non-woody part, we suggest that the vitamin may be utilized in the formation of the lignin. This hypothesis may also explain the function of the vitamin C in the stem. The concentration gradient of vitamin C in the stem declines towards the roots. In the large trees yielding our main sample we could only follow this gradient 2 or 3 ft. down the shoots into the main stem. By examining a sapling about 4 ft. high we

were able to establish the complete gradient from the buds down to the finest roots (Table 6).

Table 6 *Concentration gradient of vitamin C in walnut sapling examined March 1945*

		Vitamin C (mg/100 g)	
(O.Y.G. denotes current year's growth, L.Y.G., last year's growth)			
Buds		O.Y.G.	258
Stem cortex and phloem		L.Y.G.	252
			183
Roots			140
Fine roots			94

SUMMARY

1. Studies on samples of different tissues collected during three seasons from *Juglans regia* trees growing in several districts round London showed, in the endocarp, remarkably high concentrations of vitamin C, which may be used in lignification. This vitamin C probably originates mainly in photosynthetic centres in the leaves.

2. Examination of the intervening tissues showed that physiological conditions (acidity, concentration of enzymes and of vitamin C) militate against translocation of vitamin C as such from leaf to nut. The experimental findings favour an alternative hypothesis, which will be considered later.

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The Accuracy of the Svedberg Oil-turbine Ultracentrifuge

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1 THE SEDIMENTATION CONSTANT

The values of sedimentation constants which appear in the literature show more variation than one would expect to obtain from measurements made on well defined substances by an accurate physical method. Measurements made in Oxford on several proteins which are believed to be definite and homogeneous have always given values lower than those obtained

elsewhere (Table 1), correction of these values for errors described in the present work would further increase these discrepancies. The large difference between the values for the sedimentation constant of insulin of Gutfreund & Ogston (1946) and of Miller & Andersson (1942) finally led us to investigate the errors of our Svedberg ultracentrifuge.

Table 1 *Comparison of sedimentation constants from various sources*

Substance	Author	Concn (g/100 ml.)	S_{20} (corr) $\times 10^{13}$
Horse serum, albumin (crystallized, carbohydrate free)	Kekwick (1938) (Uppsala)	—	4.48
	(Oxford, unpublished)	1	4.31
Ovalbumin	Svedberg & Pedersen (1940)	0.5	3.55
	(Oxford, unpublished)	1	3.4
Insulin	Miller & Andersson (1942)	0	3.55
	Gutfreund & Ogston (1946)	0-1	3.34
Lactoglobulin	Pedersen (1936)	0-1	3.12
	Johnston & Ogston (1946)	1	2.83
	This work	0	2.83

Provided that sedimentation is undisturbed by mechanical vibration or by thermal convection, the accuracy with which a sedimentation constant can be determined should be limited only by the accuracy of the measurement of the following quantities (1) the positions of the sedimentation boundary at stated times, relative to the meniscus and to the centre of rotation, (2) the speed of rotation, (3) the state of the solution with respect to composition and temperature.

Since no substance is available whose sedimentation constant is certainly known, it was necessary to test for sources of inaccuracy piecemeal. Lactoglobulin was used as a working substance, since it appears to be one of the best defined and most stable proteins.

The accuracy of the measurement of the positions of the boundary and of the speed of rotation were easily checked, the chemical composition of the solution was a matter of ordinary technique, the question of cell temperature proved more complex, and, before attempting to measure it, we had to examine the factors which may affect it.

Calculation of the sedimentation constant

The traditional method of calculation is to obtain an estimate of the 'uncorrected' sedimentation constant S_{20} from the movement of the boundary during each of a succession of intervals, S_{20} is the rate of movement, reduced to unit gravitational field and corrected for the effect of temperature on the viscosity of the solvent. The effects of temperature on the density of the solvent, and of the buffer salts on its density and viscosity, do not vary appreciably during the course of a run and are, therefore, applied as corrections to the mean value \bar{S}_{20} , to give \bar{S}_{20} (corr), this is the rate of movement in water at 20° under unit gravitational field.

This procedure of averaging the estimates of S_{20} obtained in successive intervals does not, however, make nearly full statistical use of the data available. A method was therefore devised which does so.

The value of S_{20} from a given interval is given by

$$S_{20} = \frac{\Delta x}{\Delta t} \frac{\eta_{\theta}}{\eta_{20}} \frac{1}{(A - \bar{I} + \bar{x}) \omega^2}, \quad (1)$$

where Δx is the distance moved during time Δt , η_{θ} , η_{20} are the viscosities of water at θ° and 20° , A is the distance of the index from the centre of rotation, \bar{I} the mean distance of the meniscus from the index and \bar{x} the mean distance of the boundary from the meniscus, so that $(A - \bar{I} + \bar{x})$ is the mean distance of the boundary from the centre of rotation during that interval, ω is the mean angular speed of rotation. Since S_{20} is appreciably constant, and since an error of x contributes only a very small proportionate error to $(A - \bar{I} + \bar{x})$, equation (1) may be rearranged and integrated, without sensible error, to a linear equation in x .

$$x = S(\Delta x) = S_{20} S[\Delta t \eta_{20} / \eta_{\theta} (A - \bar{I} + \bar{x}) \omega^2] = S_{20} S(\Delta t') \quad (2)$$

S_{20} is then the slope of a plot of x against $S(\Delta t')$ and its value, with its standard deviation, may be calculated by the standard method of obtaining linear regression coefficients. Corrections for the effects of temperature on density and of the buffer salts are then applied as usual.

Using this method, good linear plots were obtained (Fig. 1), the standard deviation of the estimate of S_{20} seldom exceeded 0.5% (Table 2).

EXPERIMENTAL

Optical system and measurement

The optical system was the original Philpot (1938) diagonal schlieren system, modified by replacing the fused silica windows of the rotor casing and the compound quartz fluoride lenses by a pair of simple glass lenses. A considerable improvement of definition resulted from this replacement. The source of light was a 250 W Mazda box type mercury lamp used with a monochromator, this reduced exposures tenfold compared with those previously required, and caused a further sharpening of the image, since the boundary did not now move an appreciable distance during an exposure (4-10 sec).

The magnification of the spherical (schlieren) lens was measured over the whole field by photographing a finely ruled scale or its projected image, with the rotor first static and then running in the evacuated casing, the same value was obtained in each case, constant to 0.1% over the field.

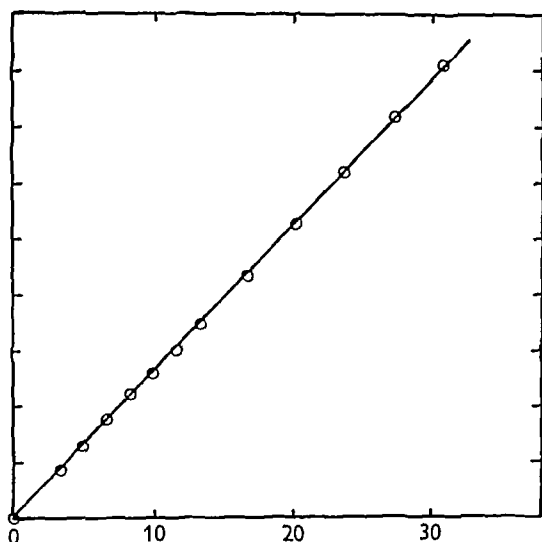


Fig 1 Plot of a run by equation (2) Ordinate $S(\Delta x)$ in cm measured on the photographic record, abscissa $S(\Delta t')$ in cm measured on the photographic record $\times 10^{-11}/\text{sec}$

Measurement of the plates was made by means of a travelling microscope reading correct to 0.001 cm. The position of the boundary was taken as the maximum of the 'peak' in the diagonal schlieren diagram, and was obtained to 0.005 cm or less, according to the thickness of the boundary.

The distance of the index hole in the balance cell from the centre of rotation was measured to 0.05%.

The speed of rotation

The Hartmann Kempf reed frequency meter, for measuring the frequency of the alternating current generated by the rotor, was supplemented with a frequency bridge designed and built for us by Mr R. P. Martin and Mr P. A. Davenport, of the Clarendon Laboratory (to be published). This enables the speed to be measured to 0.1% in 4 frequency ranges, 300 ± 5 , 450 ± 5 , 700 ± 10 and 1000 ± 10 revs/sec. The bridge reads continuously within these ranges, so that control of speed drift is made easy. The bridge was calibrated at intervals against a crystal clock. During a run, the speed was kept constant within ± 5 revs/sec and rarely changed by more than 2 revs/sec during any 10 min interval between photographs. All alterations of the turbine oil pressure were made at the beginnings of intervals, so that the drift of speed during any interval was nearly constant. The use of the bridge effected a noticeable reduction of the standard deviation of S_{20} , compared with estimates from runs in which the speed was measured by the Hartmann Kempf meter.

The cell temperature

The rotor runs, on plain journal bearings, in a casing which contains hydrogen at low pressure. Heat is generated in the bearings and by friction with the hydrogen, it is lost to the cooled turbine oil and to the casing, the casing loses heat to the turbine oil and to the surrounding air. The temperature of the rotor, and thence of the cell contents, is thus controlled by several factors, it is measured by means of a thermocouple whose junction has a clearance of 0.025 cm on the rotor surface. Svedberg & Pedersen (1940) state that this thermocouple reads the cell temperature within 0.5° . However, a change in the pressure of turbine oil, or of the pressure of hydrogen, causes so rapid a change in the reading of the thermocouple as to make it unlikely that this corresponds directly with a change of temperature of the whole mass of the rotor. It seems likely, on the contrary, that the temperature of the thermocouple, although controlled by the same factors as control that of the rotor, is not simply related to it.

Since it would be laborious to calibrate the thermocouple in terms of cell temperature, for variation of all possible factors, our aim was to find standard conditions which could be easily reproduced and then to calibrate under these conditions. A series of runs was, therefore, carried out, using a solution of lactoglobulin (1 g/100 ml) in buffer (0.1 M NaCl, 0.1 M Na acetate, 0.04 M acetic acid), to test the effects of varying conditions on the apparent sedimentation constant. The results were as follows (Table 2).

(a) In our former procedure, the cold water which cools the turbine oil was turned on at the beginning of the run; the temperature of the rotor thermocouple rose rapidly during speeding up and then, when full speed was reached, drifted upwards steadily for several hours ($0.1-0.3^\circ/10$ min). When the cooling water was not turned on until full speed had nearly been reached (20-25 min after starting), the temperature rose more rapidly at first, but reached a steady value within 50-60 min after starting (30 min after full speed), thereafter it did not change by more than 0.2° . The apparent sedimentation constants obtained by the latter procedure were significantly lower (by 1-2%) than by the former (Table 2). The new procedure was adopted as standard.

(b) For some years we have used 5 mm pressure of hydrogen in the casing, instead of the 20 mm recommended by Svedberg & Pedersen (1940), because Philpot (personal communication) had observed that there was less risk of thermal convection at the lower pressure in experiments using acetone as solvent. The quick effect of a change of hydrogen pressure on the thermocouple reading led us to test the effect of hydrogen pressure on sedimentation constant. This was found to be considerable, higher values being obtained at pressures less than 10 mm (Fig 2 and Table 2). No significant change accompanied a further raising of the pressure to 20 mm, we, therefore, adopted 10-12 mm as standard.

(c) Other variations did not appear to exert any considerable effect. The average speed of rotation was always within a few cycles of 1000 rev/sec, the room temperature varied between 19 and 22° in various runs, but there appeared to be no correlation between this and the apparent sedimentation constant, the degree of filling of the cell, the presence or absence of a layer of paraffin (lidded cells were used) and the cell thickness did not affect the result.

Table 2 S_{20} under various conditions by equation (2)

Run	Conditions				Apparent S_{20} (corr) $\times 10^{13}$	True S_{20} (corr) $\times 10^{13}$	s D of S_{20} (corr) $\times 10^{13}$
	H ₂ pressure (mm)	Rotor temp	Paraffin present	Cell (mm)			
Experiments under standard conditions							
563	10	Steady	No	12	2 876	2 813	0 007
571	10	"	"	12	2 866	2 803	0 006
559	10	"	"	12	2 899	2 835	0 009
572	10	"	"	12	2 854	2 791	0 015
574	10	"	"	12	2 858	2 795	0 012
593	10	"	"	3	2 863	2 800	0 014
532	18	"	"	12	2 873	2 810	0 006
538	20	"	Yes	12	2 871	2 808	0 008
550	20	"	No	12	2 891	2 827	0 008
					Mean of S_{20} (corr) 2 809		
					s D of mean		0 014
Variation of conditions							
558	10	Rising	No	12	2 934	—	0 009
577	10	"	"	12	2 930	—	0 010
527	5	Steady	"	12	2 917	—	0 008
530	5	"	"	12	2 929	—	0 011
578	5	"	"	12	2 927	—	0 009
533	c 1	Rising	"	12	3 042	—	0 008
536	1	"	Yes	12	2 944	—	0 016
564	1	Steady	No	12	2 959	—	0 004

(d) A number of runs done under these standard conditions (Table 2) shows a standard deviation of the estimates of S_{20} (corr) of 0.014 or 0.5%, which is little greater than the normal S.D. of the estimate from a single run. This indicates that there is no important cause of random variation operating between runs other than those operating within a given run, and thus further confirms the unimportance of such factors as mean rotor speed (within the narrow range used), cell filling and room temperature.

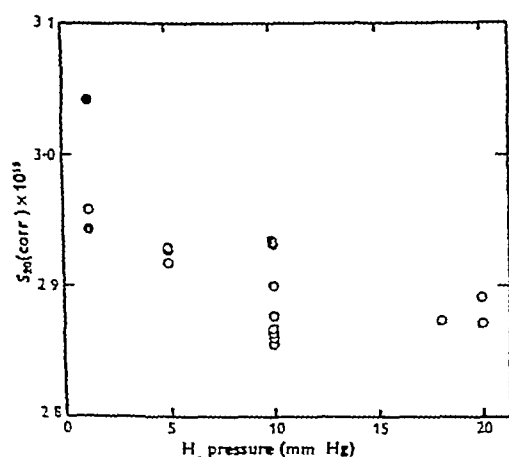


Fig. 2 Apparent S_{20} (corr) $\times 10^{13}$ against the pressure of hydrogen in mm of mercury, open circles, steady temperature, filled circles, rising temperature

(e) A calibration of the reading of the rotor thermocouple could now be undertaken. First it was shown by comparison with a National Physical Laboratory standard thermometer, to read its own temperature correct to 0.1°. Next calibration was carried out by the method given by

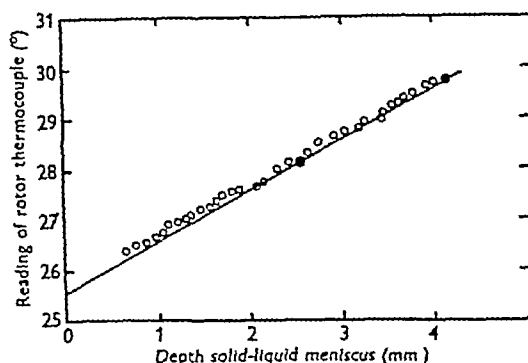


Fig. 3 Record of a melting point run. Open circles represent values obtained during upward drift of temperature, filled circles represent readings at thermal steady states

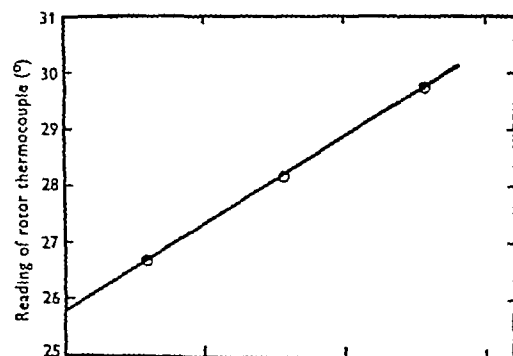


Fig. 4 Record of steady state points in two melting point runs. Abscissa proportional to the pressure at the solid liquid meniscus. Values obtained at different thermal steady states

Svedberg & Pedersen (1940) Diphenyl ether was five times recrystallized and then melted at 26.7–26.9°. A carefully cleaned 3 mm cell was filled with the melted substance, which was allowed to solidify before insertion of the cell in the rotor. After full speed was attained, a slow upward drift of the rotor temperature was allowed to take place (0.2°/10 min). When melting had begun, photographic records of the positions of the solid liquid meniscus and readings of the rotor thermocouple were taken at intervals. In order to imitate our standard conditions of thermal steady state, three such states, at different temperatures, were attained in the course of two runs. Fig. 3 shows the depths of the meniscus plotted against the thermocouple readings; it is seen that the points obtained during a drift of temperature lie 0.2–0.3° above the line drawn through the steady state points. Fig. 4 shows the thermocouple readings for the three steady states plotted against numbers proportional to the pressures at the solid liquid meniscus (the actual pressures cannot be given, since the density of the liquid was not accurately known), extrapolation to zero pressure (effectively equal to 1 atm) gives 25.8°, 1.0° below the mean melting point (Diphenyl ether removed from the cell after these runs showed an unchanged melting point). This means that the apparent sedimentation constants obtained under these conditions must be multiplied by 0.978 to give the true value.

Lactoglobulin solutions

Lactoglobulin was made from fresh milk by the method of Palmer (1934). It was twice recrystallized and stored as crystals in water under toluene in the cold room, recrystallization was effected by dissolving the crystals in NaCl

solution, removing any insoluble material adjusting the pH to 5.2 by dialysis against acetate buffer and dialyzing against water. Solutions were made by dissolving the crystals in buffer (0.1M NaCl, 0.1M Na acetate, 0.04M acetic acid) and dialyzing against buffer. The concentration of protein was estimated refractometrically against diffusate, using 0.00180 as the specific refractive increment, the solution was then diluted with diffusate to give a concentration of 1 g protein/100 ml. Solutions were kept in the cold room and did not change their sedimentation properties during storage.

DISCUSSION

This work shows that the Svedberg oil turbine ultracentrifuge can give highly reproducible results provided that the conditions of running are carefully controlled, and that proper attention is given to the measurement of all the quantities concerned. This conclusion may hold good for other types of ultracentrifuge. The reproducibility which we claim compares favourably with other published data.

We believe, also, that we have controlled all important sources of systematic error, and that the results which we now quote are accurate to 0.5% (s.d.). Our value of 2.81×10^{-13} for the sedimentation constant of lactoglobulin differs considerably from Pedersen's (1936) value of 3.12×10^{-13} . Further discussion of this discrepancy will be left to a later paper.

2 ESTIMATION OF THE QUANTITY OF A SEDIMENTING COMPONENT

Subject to the limitations found by Johnston & Ogston (1946), the amount of a sedimenting component, in terms of its refractive increment, may be estimated by measuring the area of the 'peak' of the diagonal schlieren diagram due to this component. Comparison of this with the total refractive increment of the solution due to protein (measured against diffusate) gives information about the homogeneity of the preparation. A deficiency may be due to another visible component or to material which sediments too slowly or too quickly to be observed, or which is too heterogeneous to form a visible boundary. A single homogeneous component should give, by integration of the peak, a value equal to the total refractive increment.

The accuracy of measurement of the quantity of a component by the 'scale' method of Lamm (1934) appears to be of the order of 1 to 2%, that of the diagonal schlieren method of Philpot (1938) has been believed to be rather lower. We have modified the method of estimation of Philpot's method and now find its accuracy to be nearly equal to that of Lamm.

EXPERIMENTAL

Optical system

Modifications to the optical system described above have improved the photographic definition. The use of a high intensity mercury lamp as a source of monochromatic light has resulted in a reduction of exposure times by tenfold, as a result, the boundary does not move an appreciable fraction of its thickness during an exposure, and blurring of the limbs of the 'peak' is avoided. The line of demarcation on the photographic record (Fig. 5) is not quite sharp, but shows a narrow region of shading, from black to clear, due to diffraction, however, any line of constant photographic blackening is equivalent to the theoretical line of demarcation, being merely displaced by a constant distance at right angles to the cylindrical axis of the cylindrical lens. The width of the region of shading can be narrowed by making a high contrast print from the original record, we find, however, that this procedure gives no noticeable increase in accuracy, except where the region of shading is broadened by use of the schlieren system at high optical sensitivity.

Following Johnston & Ogston (1946), we have made tracings on white paper of enlarged projections of the photographic records, using a linear magnification of 4.5. A line of constant blackening can be traced without difficulty.

Area measurements of sedimentation diagrams

The area to be measured is that included between the 'peak' due to the boundary and the base line from which this arises, base line is seen both above and below the boundary, if the boundary is clear of the meniscus and the bottom of the cell, and represents uniform concentration. For rough work, the base line may be sketched in through the boundary region, but for accurate work an experimentally determined base line must be used. The base line suffers distortion of two kinds.

(a) Curvature of the cell end plates produces a curvature of the base line (Fig 5) and sedimentation of buffer solutes may also produce a distortion. It is therefore necessary to



Fig 5 Photographic records of boundary and base line

obtain a base line, corresponding with each record of the boundary whose area it is wished to measure, using buffer of identical composition and under the same conditions of rotor speed, temperature, time (we have found this unimportant with acetate buffer), degree of filling of the cell and optical settings.

(b) The base line, obtained as described in (a), fits the boundary diagram only above the boundary (Fig 6). This

is due to the different refractive indices of the solution below the boundary in the experimental run (where it contains protein) and the base line run (where it contains only buffer). This difference, since the cell end plates are distorted to form a lens, alters the focal length of the optical system, which causes the base line to change in slope.

We have, therefore, used only diagrams in which a considerable length of base line is visible both above and below the boundary, and have fitted the tracing of the experimentally determined base line to the regions above and below the boundary, giving them the requisite slopes, and allowing intersection of the two parts of base line to occur in the middle of the boundary (Fig 6). This procedure appears to give a fair approximation of the true area.

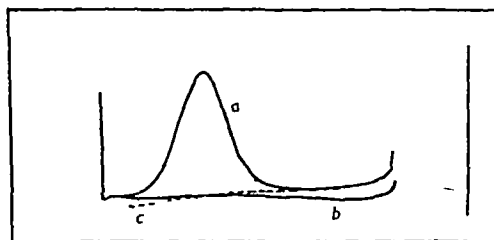


Fig 6 Tracings from photographic records (a) boundary tracing, (b) base line fitted above the boundary, (c) base line fitted below the boundary

The area between the 'peak' and the fitted base line was measured with a planimeter, making five complete circuits of the area ($P \text{ cm}^2$). The planimeter was standardized with the radius arm supplied with it, its wheel was always run on a standard paper surface. The magnification of the enlarger (M_E) was obtained by measuring the meniscus index distance on the plate by a travelling microscope and on the tracing by a ruler (to 0.5 in 120 mm). The magnification of the spherical schlieren lens (M_s) was measured as already described, and that of the cylindrical lens (M_c) by measuring the photograph through it of an accurately made rectangular aperture, the distance (a) from the focusing lens on the rotor casing to the diagonal edge (equal to its focal length) was measured by ruler (to 0.5 in 133 cm), the cell

Table 3 Measurement of boundary diagrams of lactoglobulin

(Lactoglobulin 1% total refractive increment 182×10^{-5} Cell thickness 1.219 cm)

Time from reaching full speed (min)	Angle of schlieren edge ($^\circ$ from vertical)	Estimated refractive increment $\times 10^5$ (N)	N as % of total
68	25	184	101
78	25	173	96
88	25	184	102
98	30	181	100
108	30	182	101
110	35	173	96
118	35	176	98
		Mean $179 \pm (\text{s.d.}) 4.9$	98.4 ± 2.7

(Lactoglobulin 0.526% total refractive increment 94.7×10^{-5})

Time from reaching full speed (min)	Angle of schlieren edge ($^\circ$ from vertical)	Estimated refractive increment $\times 10^5$ (N)	N as % of total
82	40	92	97
102	40	96	101
112	45	92	97
122	45	95	100
132	50	97	102
		Mean $94.4 \pm (\text{s.d.}) 2.3$	100 ± 2

thickness (b) was measured by micrometer, the thickness of the quartz plates having been measured before assembly. The refractive increment N is then given by

$$N = \frac{P \cot \psi}{5M^2_E M_s M_o ab} \left(\frac{A - I + x}{A - I} \right)^2,$$

where ψ is the angle of the diagonal edge from the vertical. The square term allows for the dilution of protein through sedimentation in a non uniform field and in a sector shaped cell (Svedberg & Pedersen, 1940).

According to this procedure, measurements have been made of the boundary diagrams obtained in two of the experiments on lactoglobulin described above. The results are given in Table 3.

DISCUSSION

The boundary diagrams obtained with lactoglobulin are ideal for accurate integration, an accuracy of about 2.5% (S.D.) was obtained from measurement of a single diagram, and greater accuracy is obtainable by taking the mean value from several diagrams of a given run (measurement of six give an accuracy of about 1%). The error will be greater than this in the following cases: (1) where the boundary is very broad, leaving too little base line for accurate fitting, (2) where it is very narrow, so that the area to be measured is small and the distance moved during an exposure is an appreciable fraction of its width, (3) where one boundary is incompletely resolved from another, or where heterogeneous material distorts the apparent base line, (4) where the use of a high optical sensitivity (with dilute solutions) lessens the sharpness of the line of demarcation in the diagram.

In the course of this work it was noticed that, for a period, wrong values had been obtained for the quantity of sedimenting components, through failure

to take into account the fact that the focusing lens then in use on the rotor casing was not placed at its focal distance from the diagonal edge. A correction of 7% is to be added to the values given in the following publications: Ogston (1945), Ogston (1946) and Johnston & Ogston (1946), application of this correction to data given in the last for serum albumin and lactoglobulin gives a mean integration value of 97%.

The accuracy of the Philpot optical system is thus shown to be little inferior to that of the scale method of Lamm (1934).

SUMMARY

1. A systematic investigation of the accuracy of measurement of sedimentation constants by means of the Svedberg oil turbine ultracentrifuge has shown that values reproducible to 0.5%, and probably accurate to the same limits, can be obtained if the conditions of running are standardized, if care is taken over measurement of the various quantities concerned, and if correction is made for error of measurement of the temperature of the cell contents. A new method of computing the sedimentation constant is given.

2. Examination of the accuracy of estimation of the quantity of a sedimenting component from the boundary diagram given by the diagonal schlieren method of Philpot (1938) has shown that, under favourable conditions, a single measurement may have a standard deviation of 2.5%, making an accuracy of 1% attainable from the data of a single run. Special attention is needed to the fitting of the base line and an improved method of doing this is described.

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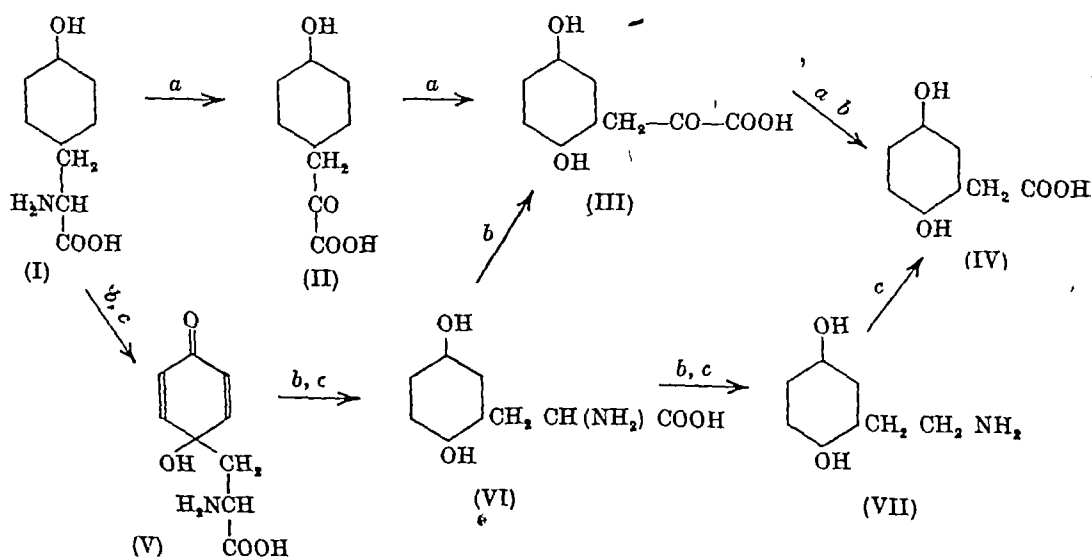
Synthesis and Resolution of 2,5-Dihydroxyphenylalanine

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It is generally believed that the main pathway of the metabolism of the amino acids phenylalanine and tyrosine in mammals involves the intermediate formation of homogentisic acid. In alcaptonuric man, and in various laboratory animals reared under certain dietary conditions, the amount of homogentisic acid excreted in the urine is proportional to the intake of the two aromatic amino acids, this indicates that the oxidation of tyrosine to a quinol with an accompanying shift of the side chain can be performed by mammals. Moreover, the recent isolation by Fishberg (1948), in cases of enterogenous cyanosis, of 1,4-benzoquinone 2-acetic acid, the quinone corresponding to homogentisic acid, has demonstrated that, even in man, the formation of compounds related to homogentisic acid is not confined to alcaptonurics and premature babies. But the assumption that such a reaction represents the main normal pathway of metabolism for these aromatic amino acids rests on an argument by analogy, and no conclusive evidence for this hypothesis has as yet been adduced. Even in alcaptonuria the exact mechanism of the conversion of tyrosine to homogentisic acid is still obscure. The oxidation of a phenol to a quinol is a fairly common biological reaction, thus small amounts of quinol are excreted on ingestion of large doses of phenol by the dog

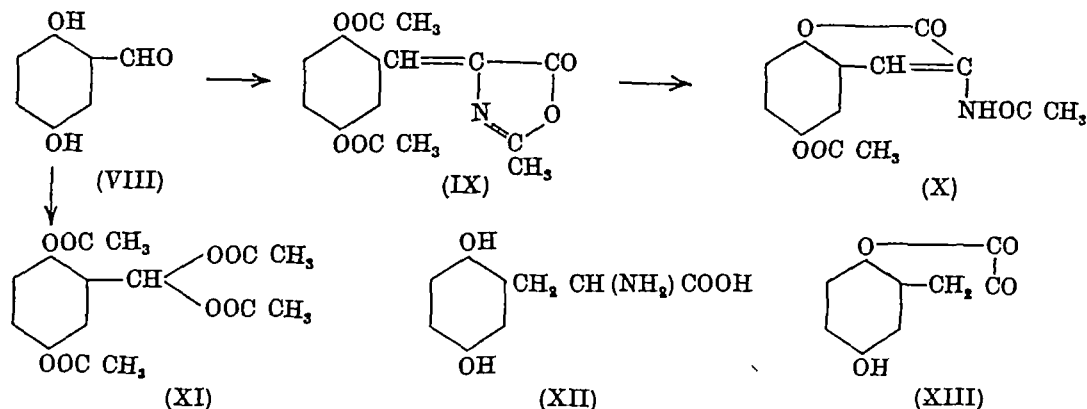
(Baumann & Preusso, 1879) and salicylic acid gives rise in man (Baldoni, 1908) and in the rat (Lutwak-Mann, 1943) to gentisic acid. But no exact biological analogy exists for the migration of a substituent originally occupying the para position. The formation of thyroxine from diiodotyrosine which occurs so readily *in vivo* and *in vitro* must involve a somewhat similar mechanism (see Pitt-Rivers, 1948). However, in the latter case, the side chain is eliminated whilst with tyrosine it is shifted to the adjacent position in the ring. It is generally assumed that tyrosine (I) is first converted to the corresponding keto acid (II) which is then further oxidized in the ring to give 2,5-dihydroxyphenylpyruvic acid (III) which on oxidative decarboxylation would yield homogentisic acid (IV). But the fact that *p*-hydroxyphenylpyruvic acid is far less effective than tyrosine as a precursor of homogentisic acid in the alcaptonuric (Neubauer, 1909, Fromherz & Hermanns, 1914) suggests that oxidation of the ring precedes that of the side chain. The small amount of extra homogentisic acid excreted on administration of *p*-hydroxyphenylpyruvic acid may be ascribed to its reconversion into tyrosine by amination. As Neubauer (1928) points out, at least two other possibilities (b, c) must be considered



Thus a second hydroxyl group might be introduced into tyrosine to give the dienone (V) which would rearrange to 2,5-dihydroxyphenylalanine (VI), this could then be oxidized through the keto acid to homogentisic acid (mechanism b) or be first decarboxylated to the amine (VII) and then oxidized (mechanism c). In order to test these possibilities it was decided to prepare and resolve 2,5-dihydroxyphenylalanine. The synthesis of 2,5-dihydroxyphenylethylamine, another possible intermediate, is described in the accompanying paper (Leaf & Neuberger, 1948).

Reaction of gentisic aldehyde with acetylglycine

The racemic amino acid was prepared in two ways. In the first synthesis gentisic aldehyde (VIII) was condensed with acetylglycine in the presence of acetic anhydride and sodium acetate. From the crude product three substances were isolated by fractional crystallization. The first compound had m.p. 140–141° and crystallized in yellow needles,



it had one fairly stable and two very labile acetyl groups. This fact, the colour and elementary analysis indicate that this is the acetylated azlactone (IX). The second substance of m.p. 226–227° is colourless and contains one labile and one stable acetyl group. It is assumed to be 3-acetamido-2-keto-6-acetoxycoumarin (X) or the tautomeric 3-acetamido-2-keto-6-acetoxychroman. Several workers have obtained both the benzamidocoumarin and the benzoylated azlactone in the condensation of salicylaldehyde and hippuric acid (Rebuffat, 1885; Plöchl & Wolfrum, 1885; Erlenmeyer & Stadlin, 1904), and similar results have been obtained in the condensation of 2,4-dihydroxybenzaldehyde and hippuric acid (Deulofeu, 1936). Dakin (1929), on the other hand, isolated only an acetylated azlactone from the condensation of salicylaldehyde and acetylglycine, and similarly no coumarin derivative was isolated in the reaction of gentisic aldehyde with hippuric acid (Neubauer & Flatow, 1907). The separation of these two products is troublesome, and attempts were, therefore, made to establish conditions under which only the azlactone would be formed. It is generally accepted that the first step in the azlactone synthesis is the formation of 2-phenyl or 2-methyloxazolone, the reactive methylene group of which then reacts with the aldehyde (see Carter, 1946). Two geometrically

isomeric azlactones will generally result, and only one of these will contain the reactive carbonyl group and the phenolic ring suitably placed for esterification to proceed. Coumarin formation thus appeared to be a secondary reaction which might be facilitated by the presence of a free hydroxyl group and by prolonged heating. It was hoped to eliminate formation of the coumarin by using 2-acetoxybenzaldehydes. However, this aim was not fully achieved. Acetoxybenzaldehydes can be readily prepared in almost quantitative yield by treating the hydroxyaldehydes with pyridine and acetic anhydride; the products are distillable crystalline substances. Condensation of 2-acetoxybenzaldehyde with hippuric acid and of 2,5-diacetoxybenzaldehyde with acetylglycine yielded mainly, but not exclusively, azlactone if the period of heating was short (30 min). If heating was continued for 1–2 hr, larger amounts of coumarin were obtained. The conclusion that even the acetylated azlactone is converted to the coumarin under the conditions of the condensation was supported by the observation that heating pure acetylated azlactone of m.p. 140° with acetic anhydride and sodium acetate at 100° produced appreciable amounts of coumarin.

The third product of the reaction was nitrogen free, and from elementary analysis and acetyl content was identified as 2,5-acetoxybenzylidene diacetate (XI). The formation of diacyl derivatives of aldehydes in the preparation of azlactones, has not, as far as we are aware, hitherto been reported, and is of particular interest in view of the suggestion of Erlenmeyer & Früstlick (1895) that such diacetates are intermediates in the formation of azlactones. It has been found, however, that the diacetates of benzaldehyde, 2-acetoxy- or 2,5-diacetoxybenzaldehyde do not form azlactones under the appropriate conditions. It seems more probable that the formation of such acyl derivatives removes aldehydes from the reaction, and explains the low yields of azlactones observed in some cases, e.g. when acetylglycine is used.

Both the coumarin (X) and the azlactone (IX) yield on hydrolysis with dilute HCl 6-hydroxy-2,3-diketochroman (XIII) which on treatment with alkali, in the absence of O₂, followed by careful acidification gives 2,5-dihydroxyphenylpyruvic acid (III). Both these compounds have already been obtained by Neubauer & Flatow (1907) by a less convenient method. On treatment with red phosphorus and HI the amino acid (XII) is obtained in good yield from either (IX) or (X). An aqueous solution of 2,5-dihydroxyphenylalanine is readily oxidized in air at a

slightly alkaline pH and slowly even at neutrality giving a melanin like pigment. Hirai (1927) claimed to have prepared the racemic amino acid by condensation of 2,5-dimethoxybenzaldehyde with glycine anhydride followed by reduction and hydrolysis. The properties of the compound obtained by the Japanese worker differ greatly, however, from those of the product described in this paper. Thus, Hirai gives a m.p. of 203–204°, whilst we found a m.p. of 235°. The amino acid obtained by us crystallizes with 1 mol of water which is not removed by drying at 15 mm pressure at room temperature over P_2O_5 . On prolonged drying at 110° the water is lost, but the anhydrous material hydrates again quickly. This property is not mentioned by Hirai. Moreover, Hirai's compound was apparently easily soluble in water, whilst we found the solubility of the racemic amino acid to be less than 1% at 18° in water. No suggestion can be offered as to the cause of this discrepancy.

Resolution of 2,5-dihydroxyphenylalanine

In the course of the work it was found that *N*-acyl derivatives of 2,5-dihydroxyphenylalanine lactonize readily, are easily oxidized, and are, therefore, not convenient for resolution. It was decided to resolve instead a suitable derivative of 2,5-dimethoxyphenylalanine and to remove the methyl groups afterwards. The 2,5-dimethoxybenzaldehyde required for this synthesis was made both by the Reimer-Tiemann method from 4-methoxyphenol followed by methylation with methyl sulphate (cf. Tiemann & Müller, 1881), and from 1,4-dimethoxybenzene by the modification of the Gattermann synthesis introduced by Adams & Levine (1923). Gulland & Virden (1928a, b) used a somewhat similar method for the preparation of this aldehyde. The 2-benzamido-3-(2',5'-dimethoxyphenyl)acrylic acid obtained from the azlactone, prepared by the condensation of the aldehyde with hippuric acid, was rather resistant to hydrogenation with a palladium or platinum catalyst (cf. Waser, 1925), but the sodium salt was readily reduced under pressure by H_2 and Raney's nickel. Attempts to resolve the resulting 2-benzamido-3-(2',5'-dimethoxyphenyl)propionic acid with brucine or quinine were unsuccessful, but (+) or (–)- α -phenylethylamine respectively yielded two pairs of diastereoisomeric salts which could be separated by fractional crystallization from water. Short hydrolysis with hydriodic acid yielded the active amino acids without any appreciable racemization. The optically active amino acid resembles the racemic compound in most of its properties, thus it also crystallized with 1 mol of water which is difficult to remove. However, the active amino acid is more insoluble in cold water than the racemic compound and can be conveniently recrystallized from that solvent. *D* and *L* configurations were assigned to the dextro and laevo rotatory amino acids respectively on the basis of the following considerations discussed in detail elsewhere (Neuberger, 1948).

(1) All *D* amino acids show a decrease in dextro or increase in laevo-rotation when the neutral molecule is transformed into the corresponding cation i.e. on addition of acid. The rotation of (+) 2,5-dihydroxyphenylalanine in water cannot be measured accurately due to low solubility. However an $[\alpha]_D$ of $+40^\circ \pm 3$ was obtained by using a 4 dm tube. In N -HCl this value is decreased to $+7.8$. The difference in molecular rotation, which is about 69 and of the same order as that found for other α amino acids containing

β aryl groups, indicates that the (–) acid has *L* configuration. Moreover, *L*-3,4-dihydroxyphenylalanine (Waser & Lewandowski, 1921) for which the *L* configuration is established by direct transformation from *L*-tyrosine has rotations similar, both in water and in acid, to those of the 2,5 compound.

(2) Another empirical and apparently generally applicable rule is that the hydantoins of *L*-amino acids have rotations which are negative and relatively large. The $[\alpha]_D$ of the hydantoin of (–) 2,5-dihydroxyphenylalanine was found to be -12.45° in aqueous ethanol.

(3) An enzyme present in guinea pig kidney decarboxylates the (–) amino acid quantitatively (Blaschko, Holton & Sloane Stanley, 1948) whilst the dextro-rotatory substance remains unchanged. This enzyme is identical with, or closely similar to, dopa decarboxylase which is specific for *L*-3,4-dihydroxyphenylalanine (Blaschko, 1942–3). Since both mammalian and bacterial decarboxylases act on *L*-amino acids only, the enzymic decarboxylation of the (–) amino acid supports strongly the conclusions drawn from the physical data, and there can be no doubt that the laevorotatory 2,5-dihydroxyphenylalanine has *L*-configuration.

The biological behaviour of 2,5-dihydroxyphenylalanine

The *L*-amino acid, if given by mouth to rats in daily doses up to 1 g, is apparently readily metabolized, no reducing substances could be detected in the urine, even after acid hydrolysis. The *D*-isomeride, too, can apparently be oxidized completely by the rat, if the dose does not exceed 200 mg/100 g body weight. Larger doses cause the excretion of small amounts of reducing material which, from its solubility, appears to be the unchanged amino acid. In normal man also, 5 g of the *L* amino acid were completely metabolized, whilst the same quantity of the *D* compound gave rise to the appearance of small amounts of a reducing substance in the urine. The amino acid appears to be completely non-toxic on oral, subcutaneous and intraperitoneal administration to rabbits, mice, rats and guinea pigs. Experiments with the racemic compound already reported (Neuberger, Rimington & Wilson, 1947) indicate that it is, at least partially, converted into homogentisic acid by the alcaptonuric. Experiments with the active amino acids will be carried out, when an opportunity arises. The findings of Blaschko *et al.* (1948) that the *L* amino acid can be decarboxylated by a mammalian enzyme have already been mentioned. The resulting 2,5-dihydroxyphenylethylamine is also readily metabolized by man and by the rat (Leaf & Neuberger, 1948). On the basis of these findings, it appears probable that the normal metabolism of tyrosine goes at least partly through the stages 2,5-dihydroxyphenylalanine and 2,5-dihydroxyphenylethylamine to homogentisic acid (cf. path c on p. 599). The alternative that the quinolic amino-acid is first oxidized to the keto acid and then decarboxylated (path b

on p 599) is less likely, since 2,5-dihydroxyphenylpyruvic acid like *p*-hydroxyphenylpyruvic acid is not a very effective precursor of homogentisic acid (Neubauer & Flatow, 1907). However, the facts presented here only indicate that the suggested pathway is possible and in accordance with known facts. The hypothesis will have to be substantiated by further experiments with isolated enzymes or tissue slices.

EXPERIMENTAL

Preparation of compounds*

Reaction of gentisic aldehyde with acetyl glycine 13.8 g of gentisic aldehyde (Neubauer & Flatow, 1907), acetyl glycine (11.7 g), anhydrous sodium acetate (9 g) and acetic anhydride (40 ml) were intimately mixed and refluxed for 2–3 min. The mixture was then heated for a further 1 hr on a water bath. The partly crystalline mixture was cooled, the solid broken up and triturated with ice water. The solid was filtered off and washed with ice water, ethanol (40 ml.) and ether. The combined mother liquors were extracted with ethyl acetate, and the ethyl acetate extracts dried and concentrated. A further crop of solid material was thus obtained. Fractional crystallization from ethanol and benzene yielded three substances. (a) A compound of m.p. 226–227°, crystallizing in needles, almost colourless and almost insoluble in cold ethanol. This substance was identified as 3-acetamido-2-keto-6-acetoxycoumarin (Found C, 59.4, H, 4.2, N, 5.5. $C_{15}H_{11}O_6N$ requires C, 59.8, H, 4.2, N, 5.36%). Labile acetyl groups were estimated as follows: 0.261 g of the material was dissolved in 25 ml. 0.1N NaOH and allowed to stand at room temperature for 2 hr, 25 ml. 0.1N HCl were then added and the mixture was steam distilled. The distillate required 10.2 ml. 0.1N NaOH for neutralization to cresol red, this gives a labile acetyl content of 16.8%. $C_{15}H_{11}O_6N$ requires a labile acetyl content of 16.4%. Total acetyl content was estimated as follows: 0.261 g of the material was refluxed for 4 hr with 20 ml of 3N H_2SO_4 and then steam distilled. On titrating the distillate with 0.1N NaOH, using cresol red as indicator, 20.5 ml were required, hence total acetyl content = 33.8% and by difference stable acetyl content = 17.0%. $C_{15}H_{11}O_6N$ requires 16.4%. The analytical results and the lack of colour leave no doubt that the structure assigned to this compound is correct. (b) The second compound was more soluble in ethanol and fairly soluble in hot benzene. It crystallized in small bright yellow needles and had m.p. 140–141°. It was identified as 2-methyl-4-(2'-5'-diacetoxybenzylidene)oxazolone (Found C, 58.9, H, 4.55, N, 4.7. $C_{15}H_{13}O_6N$ requires C, 59.4, H, 4.3, N, 4.6%). Labile and stable acetyl groups were estimated as described above (Found labile acetyl, 28.8, stable acetyl, 14.4. $C_{15}H_{13}O_6N$ requires labile acetyl, 28.4, stable acetyl, 12.2%). (c) The third compound was more soluble in ethanol and benzene than the other two. It was colourless, crystallized in tablets of m.p. 127 and did not contain N. It was identified as 2,5-diacetoxybenzylidene diacetate (Found C, 55.7, H, 5.14. $C_{15}H_{11}O_8$ requires C, 55.55, H, 4.9%). Mixed m.p. with a sample prepared by an independent method (see below) was 127°. The crystalline products were isolated in a yield of altogether 35–40%. The crude material consisted largely of the azlactone and was obtained in a yield of about 50%.

* All melting points are uncorrected.

Preparation of aldehyde diacetates and acetoxyaldehydes

2-Acetoxybenzaldehyde Salicylaldehyde (18.3 g) was added to dry pyridine (40 ml), the solution was cooled to –10° and acetic anhydride (15 g) added over 5 min. The mixture was left for 2 hr and then poured into ice water. An oil precipitated which soon crystallized. The material was filtered off, washed with ice water and dried. Yield was 90% of the theoretical. The solid was distilled at 142° (15 mm.), m.p. was 36–37°. Perkin (1868) gives m.p. 37°.

2,5-Diacetoxybenzaldehyde. 2,5-Dihydroxybenzaldehyde (6.58 g) was acetylated with pyridine (15 ml.) and acetic anhydride (9.5 ml.) as described above. The material, which crystallized on pouring the solution into water, was filtered off and dried. Yield of 2,5-diacetoxybenzaldehyde was 95%. 10 g were recrystallized from ligroin (200 ml.) and had m.p. 68°, on further recrystallization the m.p. was raised to 68.5–69° (Found C, 59.2, H, 4.7. $C_{14}H_{10}O_6$ requires C, 59.4, H, 4.5%).

2,5-Diacetoxybenzylidene diacetate 2,5-Diacetoxybenzaldehyde (2.2 g) was dissolved in acetic anhydride (5 ml), the mixture was cooled to –5° and conc. H_2SO_4 (0.05 ml.) was added. Crystals appeared almost at once. After 1 hr at 0° the mixture was poured into water containing $NaHCO_3$ (15 g) and allowed to stand at 0° for 1 hr. The crystals were then filtered off and dried. Recrystallized from ethanol, the substance had m.p. 128.5°. Yield was 90% of the theoretical. The diacetates of benzaldehyde and salicylaldehyde were prepared in a similar manner and were found to have the correct m.p.'s.

Both benzylidene diacetate and 2-acetoxybenzylidene diacetate were heated for 1 hr on a water bath with 1 equiv of hippuric acid, 1 equiv of anhydrous sodium acetate and 4 equiv of acetic anhydride. No azlactones could be isolated from these mixtures.

Azlactone formation with acetoxyaldehydes (a) 2-Acetoxybenzaldehyde and hippuric acid. A mixture of 2-acetoxybenzaldehyde (25.7 g), hippuric acid (28 g), anhydrous sodium acetate (12.7 g) and acetic anhydride (80 ml) was heated on the water bath for 20 min. The solution was cooled, poured into ice water containing ethanol (50 ml). The crystalline solid was filtered off and dried. It weighed 50 g. Fractional crystallization by the method of Asahina (1930) gave 4.5 g of pure coumarin, whilst the rest of the material consisted almost entirely of the azlactone. A longer period of heating or use of salicylaldehyde instead of the acetyl derivative decreased the crude yield and increased the proportion of the coumarin. (b) 2,5-Diacetoxybenzaldehyde and acetyl glycine. A mixture of 2,5-diacetoxybenzaldehyde (11.1 g), acetyl glycine (5.8 g), sodium acetate (4.5 g) and acetic anhydride (25 ml.) was refluxed for 10 min and then heated on the water bath for a further 0.5 hr. Working up as described above gave a 60% yield of the acetylated azlactone contaminated with some coumarin. The yield was slightly better, and the product less heterogeneous than that prepared from 2,5-dihydroxybenzaldehyde.

Preparation of DL-2,5-dihydroxyphenylalanine and 2,5-dihydroxyphenylpyruvic acid

2,5-Dihydroxyphenylpyruvic acid The keto acid and the amino acid have been prepared from the corresponding 2-methylloxazolone, the coumarin or the crude mixture

Yields from the pure compounds were higher. Only the hydrolysis of the azlactone needs to be described in detail. 5 g of the 2-methyloxazolone were refluxed with a mixture of equal parts 3*N* HCl and glacial acetic acid for 5 hr. The solution was concentrated *in vacuo*, and the lactone of the keto acid was obtained in a yield of 80%. After recrystallization from water and treatment with charcoal the m.p. of the now colourless crystals was 225°. Neubauer & Flatow (1907) state that the m.p. is above 220° and not sharp. The lactone was converted to the free keto acid as described by these authors. It had all the properties described by them.

DL-2,5-Dihydroxyphenylalanine 2-Methyl-4-(2',5'-diacetoxybenzylidene) oxazolone (10 g) was refluxed with a mixture of glacial acetic acid (60 ml), red phosphorus (2 g) and 20 ml. HI (sp. gr. 1.7) for 1 hr. The solution was filtered hot and concentrated under reduced pressure to dryness. The residue was taken up in water and extracted with ether. The aqueous solution was again evaporated to dryness and the solid dissolved in water, 20% (w/v). Lead acetate was then added carefully, until the pH was about 1.8–2.0. If the pH is allowed to rise to 3.0 and above, the Pb complex of the amino acid is precipitated. PbI₂ was then filtered off and the solution treated with H₂S. The precipitate was filtered off, and the solution concentrated *in vacuo* under N₂ to low bulk. On addition of pyridine to pH 5.0 the amino acid crystallized out. It was recrystallized from water containing a trace of SO₂. Yield of recrystallized material was on the average 50%. A further 10–20% could be recovered from the mother liquors. M.p. was 235°. The substance crystallizes with 1 mol. of water which was not removed by drying over P₂O₅ at room temperature at 10 mm pressure. On prolonged drying at 110° and at 1 mm, the water is lost. Loss of weight on drying 7.9%, C₁₅H₁₃O₅N requires 8.4%. The amino acid was analyzed as the hydrate (Found C, 49.9, H, 6.0, N, 6.5. C₁₅H₁₁O₄N·H₂O requires C, 50.2, H, 6.1, N, 6.5%). The substance gave a positive ninhydrin reaction, reduced silver nitrate slowly at pH 4–5 and instantaneously at alkaline reaction and reduced phosphomolybdic acid in acid solution.

Alternative method for the synthesis of DL-2,5-dihydroxyphenylalanine

2,5-Dimethoxybenzaldehyde This was prepared by two methods. (a) Gattermann synthesis. The method described here is shorter and more convenient than that of Gulland & Virden (1928a, b). Into a three-necked flask fitted with a mercury sealed stirrer, a reflux condenser and an inlet tube was placed 1.4 dimethoxybenzene (30 g), dry benzene (90 ml) and zinc cyanide (40.4 g). The mixture was cooled in ice and dry HCl was passed in, whilst the mixture was rapidly stirred, until saturated with HCl. Finely powdered AlCl₃ (44 g) was then added and the temperature raised to 45°. The mixture was kept at that temperature for 3.5 hr, whilst a slow stream of HCl was passed in. The mixture was then poured into 3*N* HCl (500 ml.), refluxed for 0.5 hr and cooled. Ethyl acetate (200 ml) was added, the organic layer separated, and the aqueous solution again extracted with ethyl acetate. The combined extracts were then dried, the solvent removed and the remaining oil distilled. A small amount of unchanged dimethoxybenzene came over below 130°, whilst the bulk of the material distilled sharply at 154°

(18 mm). It crystallized in the receiver and had m.p. 53°. Yield was 73% of the theoretical. (b) Reimer-Tiemann synthesis. NaOH (80 g) was dissolved in water (100 ml). To this solution, which was kept at 65–70°, was added 33 g of *p*-methoxyphenol (Robinson & Smith, 1926) and chloroform (20 g). The solution was kept at 70° by alternate cooling and warming. When the reaction had subsided a second lot of chloroform (20 g) was added, and after 10 min a third lot. The mixture was then refluxed for 1 hr, excess chloroform was removed by distillation and the solution acidified with 5*N* H₂SO₄. The aldehyde was then distilled in steam, 1.5–2 l. of distillate being collected. The distillate was extracted with ether and concentrated, the resulting oil was distilled *in vacuo* under N₂. B.p. was 144.5° (20 mm). The crude aldehyde (21 g) was methylated with methyl sulphate (1.2 equiv.) and 2*N* NaOH (1.2 equiv.) at 65°. The aldehyde after cooling was filtered off and the mother liquor extracted with ethyl acetate. The crystalline material and the oil from the ethyl acetate extraction were combined and distilled. B.p. was 180° (20 mm). Overall yield was 35–40%.

2-Phenyl-4-(2',5'-dimethylbenzylidene) oxazolone and DL-2,5-dihydroxyphenylalanine A mixture of 2,5-dimethoxybenzaldehyde (15 g), hippuric acid (16.2 g), anhydrous sodium acetate (7.4 g) and acetic anhydride (50 ml.) was heated on a water bath for 1.5 hr. The mixture was cooled to 0° and ethanol (50 ml.) added slowly. After standing for 1 hr at 0° it was poured into ice water (250 ml) and left at 0° for 2 hr. The crystalline solid was filtered off, washed with water, ethanol and ether. Yield was 75%. M.p. was 169–170°. After recrystallization from benzene m.p. was 172°. Gulland & Virden (1928b) gave m.p. 170–172° (Found C, 69.8, H, 5.00, N, 4.42. Calc. for C₁₈H₁₅O₃N C, 69.9, H, 4.85, N, 4.53%). The azlactone could be converted directly to DL-2,5-dihydroxyphenylalanine. 40 g of the azlactone were added to a mixture of glacial acetic acid (160 ml.), red phosphorus (10 g) and 125 ml. HI (sp. gr. 1.7). The mixture was refluxed for 15 min, the methyl iodide which had formed was distilled off and refluxing continued for another 2 hr. Isolation was carried out as described above. This method is more convenient for the preparation of the amino acid than the one described above, using acetylglycine. However, it cannot be used for the preparation of the keto acid.

Resolution

2-Benzamido-3-(2',5'-dimethoxyphenyl) acrylic acid This substance has already been described by Gulland & Virden (1928b), who found m.p. 195–196°. Such material, though it analyzes correctly, was found on reduction to give an acid which was not satisfactory for resolution, and it was necessary to recrystallize several times before reduction. The azlactone (30.9 g) was partially dissolved in ethanol (400 ml.) and 0.5*N* NaOH (230 ml) was then added to the boiling solution which was left at 90° for 20 min. The solution was cooled in ice and acidified with 2*N* HCl (80 ml.). The crystalline precipitate was filtered off and washed with cold water. Yield was 90%. The acid was recrystallized first from 50% (v/v) ethanol, twice from 50% (v/v) acetic acid and finally from chloroform and ligroin. The crude product had m.p. 195° as described by Gulland & Virden (1928b), but after repeated recrystallization it was 188° (Found C, 65.9, H, 5.1, N, 4.2. Calc. for C₁₈H₁₇O₅N

C, 66.1, H, 5.2, N, 4.3%) The lowering of m p on recrystallization may be associated with the possibility of *cis trans* isomerism in the acrylic acid

DL-2 Benzamido-3 (2' 5'-dimethoxyphenyl) propionic acid 75 g of the acrylic acid were dissolved in *N* NaOH (250 ml), Raney nickel suspension (20 g) was added and the vol made up to 1 l. Reduction occurred quickly at an initial pressure of 80 atm at 15°. When the reduction had stopped the solution was filtered and acidified. Yield was almost quantitative. The *benzamidopropionic acid* was recrystallized from 30% (v/v) acetic acid. It had m p 176.5° (Found C, 65.5, H, 5.9, N, 4.3 C₁₅H₁₃O₅N requires C, 65.7, H, 5.8, N, 4.4%)

(-) *Phenylethylamine salt of L-2-benzamido-3 (2' 5'-dimethoxyphenyl) propionic acid* The racemic acid (49 g) and (-) phenylethylamine (18.8 g) were dissolved in methanol (300 ml.) and the solution evaporated to dryness. The crystalline residue was dissolved in boiling water (1500 ml) and allowed to cool to 15°. The crystalline solid was filtered off, and again dissolved in water (1500 ml.) and cooled to 15°. A third recrystallization was done from 2 l of water. Rotation and m p had by then become constant. Yield was 90% of the theoretical (31.5 g). M.p was 180°, $[\alpha]_D^{18} -13.7^\circ$ in methanol (c, 2.0). The salt crystallized as a *monohydrate*. (Found C, 66.4, H, 6.8, N, 6.0 C₁₄H₂₀O₅N₂ · H₂O requires C, 66.7, H, 6.8, N, 5.7%)

(-) *Phenylethylamine salt of D-2-benzamido-3 (2' 5'-dimethoxyphenyl) propionic acid* The mother liquors amounting to 5 l. were combined and concentrated under reduced pressure (100 mm.) to about 1 l. Small amounts of crystalline material which separated out during the concentration, and which consisted of mixtures of diastereoisomers, were collected and preserved for a later resolution. The solution was then cooled to 0°, and the crystalline precipitate of m p 160° filtered off, a further crop was obtained by concentrating the solution to 300 ml. and cooling to 0°. The combined solids were again recrystallized first from water and then from ethanol. The yield was 24 g. The mother liquors were combined and concentrated. M.p was 167–168°, $[\alpha]_D^{18} +9.1^\circ$ in methanol (c, 2.0). This salt also crystallized as *monohydrate*. (Found C, 66.3, H, 6.5, N, 5.9 C₁₄H₂₀O₅N₂ · H₂O requires C, 66.7, H, 6.8, N, 6.0%)

The two salts of (+) phenylethylamine were also prepared and had the expected properties. +D salt m p 180°, $[\alpha]_D^{19} +13.8^\circ$ in methanol (c, 2.0), +L salt m p 166°, $[\alpha]_D^{19} -9.0^\circ$ in methanol (c, 2.0). The -D and +L salts were probably optically not completely pure, but further recrystallization did not raise the rotation or m p, any further.

D and L-2 Benzamido-3 (2' 5'-dimethoxyphenyl) propionic acids The -L- and +D salts respectively were decomposed as follows. 45 g of the salt were dissolved in hot water (1 l.) and 5N NaOH (50 ml) was added, whilst the solution was shaken. The phenylethylamine was removed by repeated extraction with chloroform and recovered in the usual manner. The aqueous solution was acidified with 5N HCl (55 ml.) and the precipitate filtered off and dried. The material was recrystallized first from chloroform light petroleum (b p 120°) and then from 30% (v/v) acetic acid. M p of both compounds was 170°. The L-acid had $[\alpha]_D^{17} -32.5^\circ$ in ethanol (c, 2.0), whilst the D-acid had $[\alpha]_D^{18} +32.8^\circ$ in ethanol (c, 2.0). The L-acid only was analyzed. (Found

C, 65.6, H, 5.9, N, 4.1 C₁₅H₁₃O₅N requires C, 65.7, H, 5.9, N, 4.25%) The +L and -D salts were decomposed in similar manner. The corresponding D and L-benzamido acids had m p 167 and 168° respectively and the following rotations. D acid $[\alpha]_D^{17} +30.5^\circ$ in ethanol (c, 2.0), L-acid $[\alpha]_D^{17} -31.0^\circ$ in ethanol (c, 2.0).

D and L-2,5 Dihydroxyphenylalanine. 10 g of the active benzoyl compound were hydrolyzed with 80 ml. of a mixture of equal parts of HI (sp gr 1.7) and glacial acetic acid which had previously been refluxed for 15 min with red P (0.4 g) and filtered. The mixture was refluxed for 15 min, methyl iodide removed by distillation, and the hydrolysis continued for another 1.25 hr. The amino acids were isolated as described above. Yield of recrystallized amino acid was 65–70%. The L-amino acid had m p 265°, $[\alpha]_D^{17} -8.1^\circ$ in *N* HCl (c, 2.0). (Found C, 50.1, H, 6.2, N, 6.45, C₉H₁₁O₄N · H₂O requires C, 50.2, H, 6.1, N, 6.5%) Loss of weight on drying at 110° (1 mm) 8.1%, the *monohydrate* requires 8.4%. The D amino-acid had m p 265°, $[\alpha]_D^{18} +7.9^\circ$ in *N* HCl (c, 3.0), $[\alpha]_D^{16} +40.0^\circ \pm 3.0$ in water (c, 0.15).

Hydantoin The L-amino acid was converted into the L-hydantoin, 0.5 g of the amino acid and KCNO (0.5 g) were dissolved in hot water (30 ml.) The solution was heated on the water bath for 40 min, whilst H₂ was passed through the solution. 5N HCl (7.5 ml.) was then added and heating continued for 2 hr. On cooling crystals appeared which were filtered off and, after treatment with charcoal, recrystallized from water. The L-hydantoin had m p 220–221°, $[\alpha]_D^{16} -124.5^\circ$ in 90% (v/v) ethanol (c, 0.92). (Found N, 12.3 C₁₀H₁₀O₄N₂ requires N, 12.6%)

METABOLIC EXPERIMENTS

Rats Male and female rats of the Institute stock, weighing between 250 and 300 g, and fed ordinary rations, were used for these experiments. The amino-acids were mixed with the food, and allowance was made for material which had not been consumed. The analytical methods used have been described (Neuberger, 1947). The L-2,5 dihydroxyphenylalanine was given to 10 rats in doses ranging from 50 to 350 mg/100 g body wt/day. Only two animals, receiving the highest doses, excreted a reducing substance which appeared to be unchanged amino acid. It amounted to 8 and 10% respectively of the intake, as judged by the Briggs reaction. Identical results were obtained with urine samples which had been hydrolyzed with 0.5N or 1.0N HCl for 1 hr at 100°. The D amino acid gave rise to the excretion of reducing substances in four animals which had received 250, 275, 300 and 325 mg/100 g body wt/day. No reducing substance was excreted with doses of 100 and 150 mg/100 body wt/day. The amount of material excreted, as estimated by the Briggs reaction, amounted to 10, 12, 20 and 18% respectively of the intake. Hydrolysis with HCl, as described above, did not increase the amounts of reducing material.

Man The L amino acid (5 g) dissolved in warm water (500 ml) was taken by a subject (A N) and urine collected for the next 48 hr. No reducing material could be detected in the urine, even after hydrolysis by acid. The same amount of the D amino acid produced reducing material in the urine collected during the first 8 hr after ingestion. It amounted to about 0.8 g. A similar result was obtained on ingestion of 3 g of the D amino acid, 0.6 g of reducing material (in terms of the amino acid) was excreted. No untoward symptoms were experienced.

Toxicity The low solubility of the active amino acid in water precluded the use of very large amounts of the compound in tests using a parenteral route. A cat (2.1 kg) was given 30 ml of a supersaturated 2.5% solution of the DL amino acid by stomach tube. No toxic symptoms were observed. Eight mice (20–30 g wt) were injected intraperitoneally with 0.15 ml each of 0.5% solution of the L amino acid, no toxic symptoms were observed. Similar negative results were obtained on intravenous administration to rats and intraperitoneal administration to guinea pigs and rats.

SUMMARY

1. Gentisic aldehyde has been condensed with acetyl glycine to give a mixture of an azlactone and a coumarin derivative. Both compounds have been converted to 2,5 dihydroxyphenylalanine and the corresponding α keto acid.

2. The quinolic amino acid has also been prepared from 2,5 dimethoxybenzaldehyde through the azlactone and the benzamidopropionic acid. The latter has been resolved with phenylethylamine and the two optical isomers of the free amino acid been obtained.

3. It has been shown that both isomers are well metabolized by man and rat, the D compound only slightly less efficiently than the L isomeride. Both substances were shown to be non toxic. Reasons are advanced for the assumption that L 2,5 dihydroxyphenylalanine is an intermediate in tyrosine metabolism.

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The Preparation of Homogentisic Acid and of 2:5-Dihydroxyphenylethylamine

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In this paper is reported a convenient method for the preparation of homogentisic acid and a synthesis of 2:5-dihydroxyphenylethylamine. This amine is of interest as a potential intermediate in tyrosine metabolism in mammals (Neuberger, 1948) and also as a pressor substance. We have also included a few observations relating to the biological behaviour of these two compounds.

Preparation of homogentisic acid

This acid was synthesized soon after its discovery in alcaptonuric urine by Baumann & Fraenkel (1894) from 2:5-dimethoxybenzaldehyde by successive conversion to the alcohol, chloride and nitrile, followed by hydrolysis. The overall yield was very poor, due mainly to side reactions occurring in the conversion of the chloride to the nitrile. Schoepf & Winterhalter (1940), who studied systematically the formation of substituted phenylacetic acids by various methods, have found that a large excess of cyanide has to be used in such reactions if a good yield of the nitrile is to be obtained. We hoped that the chloride might be conveniently prepared by chloromethylation of 1:4-dimethoxybenzene, however, even under the most varied conditions, only the dichloromethyl compound could be obtained. Since, as will be shown later, homogentisic acid can be prepared from 2:5-dimethoxybenzaldehyde in good overall yield by another method, we did not explore the method of Baumann & Fraenkel (1894) any further.

Various other methods for the synthesis of homogentisic acid have been described, but the yields reported were in most cases very poor. Hahn & Stenner (1929) prepared 2:5-dibenzoyloxy-1-allylbenzene from hydroquinone, and claimed to have oxidized the allyl compound directly with very dilute ozone to benzoylhomogentisic acid in good yield. In our hands the method was not found to be very satisfactory. The benzoyl groups are very labile, and the material had, as Hahn & Stenner themselves found, to be rebenzoylated during the synthesis. Owing to the low concentration of ozone to be employed, the oxidation has to be carried out over many hours or even days, if moderately large amounts of material are used (cf. Schoepf & Winterhalter, 1940). Moreover, apart from homogentisic acid, other substances are produced in the oxidation. Hill & Short (1937) obtained good yields in the oxidation of 3-allyl-*o*-tolyl methyl ether with aqueous KMnO_4 containing the required amount of acetic acid. We have applied their method to the oxidation of 1:4-dimethoxy-2-allylbenzene. This compound is readily obtained by Claisen rearrangement from 1-methoxy-4-allyloxybenzene (Mauthner, 1921) followed by methylation. However, oxidation of this compound with KMnO_4 gave, under the many conditions which were tried, a

mixture of substances, and the yield of the desired 2:5-dimethoxyphenylacetic acid was generally poor. Apart from 2:5-dimethoxyphenylbenzoic acid, a large amount of a neutral compound was obtained which was identified as 3-(2':5'-dimethoxyphenyl)propane-1,2-diol. This glycol reacted with periodic acid to give formaldehyde and 2:5-dimethoxyphenylacetaldehyde which was not isolated, but oxidized directly to the corresponding acid. The diol was also prepared, albeit in poor yield, by oxidizing the allyl compound with performic acid (Swern, Billen & Scanlan, 1946; English & Gregory, 1947). Oxidation of *o*-hydroxyphenylacetic acid with persulphate gave homogentisic acid, but again the yield was poor.

Homogentisic acid was finally prepared in good yield by oxidation of 2:5-dimethoxyphenylpyruvic acid with H_2O_2 , followed by hydrolysis. 2:5-Dimethoxybenzaldehyde was condensed with hippuric acid (Gulland & Virden, 1928; Neuberger, 1948) and the azlactone ring opened with alkali. Gulland & Virden (1928) then hydrolyzed the benzamidoacrylic acid and separated the keto and benzoic acids with SO_2 . It was found more convenient to oxidize the mixture of the two acids directly, and to separate the benzoic and 2:5-dimethoxyphenylacetic acids by esterification and fractional distillation (see Snyder, Buck & Ide, 1943). Short-hydrolysis with HBr yielded homogentisic acid in good overall yield. Two other methods have been published which might be equally suitable for the preparation of this acid. Schwenk & Bloch (1942) have prepared 2:5-dimethoxyphenylacetic acid by a modification of the Willgerodt reaction from the corresponding acetophenone and recently McElvain & Engelhardt (1944) have obtained the lactone of homogentisic acid by hydrolysis of 5-hydroxy-2-ethoxycoumarone which had been made by condensation of *p*-quinone with ketene acetal.

Synthesis of 2:5-dihydroxyphenylethylamine

The corresponding dimethoxy compound has been prepared by a Hofmann degradation of 3-(2':5'-dimethoxyphenyl)propionamide (Buck, 1932), and also by electrolytic reduction of 2:5-dimethoxy- ω -nitrostyrene (Sugasawa & Shigehara, 1941), the former method giving the better yield. In the present work the Curtius degradation was used. The propionic acid was made as described by Buck (1932), except that the cinnamic acid was reduced by H_2 under pressure, using Raney's nickel as catalyst. The propionic acid was esterified by the method of Baker, Querry, Saffir & Bernstein (1947) and converted into the hydrazide and azide. Decomposition of the latter gave 2:5-dimethoxyphenylethylamine in excellent overall yield and on further treatment with HI , 2:5-dihydroxyphenylethylamine was obtained as the hydriodide which was converted with AgCl to the hydrochloride. The amine is easily oxidized by air,

even in acid solution. It is much more sensitive to O_2 than the corresponding catechol derivative which was prepared for comparison. The free base, which is not very soluble in water, has been obtained crystalline, but appears to be unstable even in the complete absence of O_2 .

Biological observations

Homogentisic acid It has been reported (Neuberger, Rimington & Wilson, 1947) that the blood level of homogentisic acid in the human alcaptonuria is vanishingly low at a time when the concentration in the urine is quite high. Various explanations for this finding were discussed. We have not been able to carry out further investigations on alcaptonurics, but had to confine ourselves to normal subjects. Ingestion of homogentisic acid (H.A.) in quantities up to 5 g did not produce alcaptonuria, nor a measurable increase of reducing substances in the blood. Intravenous injection, however, of quantities of 0.3–1.0 g produced a marked alcaptonuria which lasted, at the most, for only 40 min. The total amount of H.A. excreted varied between 28 and 64% of the administered dose, the rest being presumably metabolized. The blood level of H.A. was, in all experiments, well below 15 mg/100 ml, and in some cases of the order of 2–5 mg/100 ml. The accuracy of the estimation of H.A. in serum or plasma is low with present methods (Neuberger *et al.* 1947), especially with the very small quantities involved, and no quantitative significance can be attached to the plasma figures. Nevertheless, it is possible to arrive at certain conclusions. In several experiments, e.g. 0.3 g H.A. was injected, and on the average a third of the injected dose was recovered in the urine. After H.A. had completely mixed with the blood, the concentration in the whole blood could not have been greater than 6 mg/100 ml, if the blood volume is assumed to be about 5 l. If allowance is made for oxidation in the body and diffusion into the intercellular space, the concentration was probably appreciably lower. The values found, 1.5–3.8 mg/100 ml, are therefore quite reasonable. It thus appears that, even in normal man, the kidney threshold for H.A. is well below 4 mg/100 ml. In normals alcaptonuria cannot be produced by feeding large quantities of tyrosine by mouth, and it must therefore be assumed that H.A., if it is an intermediate, never appears in the blood in appreciable amounts, and is presumably rapidly oxidized in the organ in which it is first formed. The alcaptonuric has probably the same low kidney threshold as the normal, but he excretes H.A. in high concentrations in the urine, though the acid is either absent from the blood or present in minute amounts only. A reasonable explanation of these findings is that H.A. is formed and normally further metabolized in the kidney. No accumulation of H.A. occurs since its destruction is faster than its formation. In experi-

mental alcaptonuria this destruction may be slowed down somewhat, some H.A. accumulates and is excreted owing to its low threshold. In human alcaptonuria, the inhibition of further oxidation may be more complete, and consequently a much larger proportion of H.A. is eliminated in the urine.

2,5-Dihydroxyphenylethylamine This amine is a pressor substance of somewhat weaker potency than 3,4-dihydroxyphenylethylamine. A more detailed study of the pharmacological properties of the new amine has been undertaken in the Pharmacological Department, University of Oxford, and will be published elsewhere. The 2,5-dihydroxyphenylethylamine is toxic, if given by vein, to the mouse, a dose of 125 mg/kg body weight was found to be lethal. But given orally, the toxicity is low, at least in the rat. Thus 200–300 mg/day were tolerated quite well by adult animals (300 g). The amine appears to be readily metabolized since the administration of such large doses did not produce any reducing material in the urine. Moreover, no hydroquinone derivatives could be detected after treatment of the urine with acid. Thus four derivatives of hydroquinone, viz. 2,5-dihydroxyphenylalanine (Neuberger, 1948), the corresponding ethylamine and the acetic and pyruvic acids are all readily metabolized by the rat. This is in marked contrast to the behaviour of similar derivatives of catechol. 3,4-Dihydroxyphenylalanine is only incompletely metabolized by man (Guggenheim, 1913), the rabbit (Fromherz & Hermanns, 1914) and the rat (Holtz & Credner, 1944), and the same applies to 3,4-dihydroxyphenyl ethylamine, as far as the rat is concerned. In the present work it was found that 3,4-dihydroxyphenylacetic acid is largely excreted unchanged by the rat, even if only relatively small doses are given. Homogentisic acid, on the other hand, is readily metabolized. It thus appears that the mammalian organism is much better adapted to the oxidation of hydroquinone than of catechol derivatives, and this supports the conclusion that the former are normal intermediates in metabolism.

EXPERIMENTAL

Preparation of compounds

1,4-Dimethoxy 2,5-di(chloromethyl) benzene. A mixture of 1,4-dimethoxybenzene (69 g), 40% formaldehyde solution (37.5 ml.) and 36% (w/v) HCl (350 ml) was stirred for 6 hr at 55°, whilst a slow stream of HCl was passed through the solution. The product was taken up in ethyl acetate, washed thoroughly with water and the ethyl acetate solution dried and concentrated. Distillation of the remaining oil *in vacuo* (0.1 mm) gave unchanged 1,4-dimethoxybenzene (32 g) and the dichloromethyl ether (34 g). The m.p. of the latter was 165–166° (after recrystallization from benzene). This substance has previously been prepared by I.G. Farbenindustrie (British Patent no. 347,892) and is

stated to have m p 165°. Variation of the conditions such as reducing the reaction time and temperature did not appreciably affect the result

Oxidation of 1 4-dimethoxy 2 allylbenzene

Oxidation with permanganate in acetone To a solution of 1 4-dimethoxy 2 allylbenzene (17.4 g) in acetone (100 ml.) was added over a period of 2 hr KMnO_4 (52 g) in 70% (v/v) acetone (800 ml). The solution was stirred and the temperature kept at 25–30°, the MnO_2 which was filtered off was extracted three times with boiling water (150 ml each), and the combined solutions treated with SO_2 till colourless. The solution was then made alkaline and extracted with ether, this extract is the 'neutral' fraction. The aqueous solution was then made acid to congo red, concentrated *in vacuo* to low vol and again extracted exhaustively with ether. The 'acidic' ethereal solution was concentrated to dryness, and the residue taken up in hot water, treated with charcoal and cooled. The crystalline material was collected and recrystallized from water. It had m p 125° and an acid equiv of 194, calc for $\text{C}_{16}\text{H}_{18}\text{O}_4$ 196. Wolkow & Baumann (1891) give m p of 2 5 dimethoxyphenylacetic acid 124.5°. The mother liquor gave a further crop of crystalline material, fractional crystallization of which gave some more of the acetic acid and also 2 5 dimethoxyphenylbenzoic acid of m p 76°, the latter on hydrolysis gave gentisic acid. The yield of 2 5 dimethoxyphenylacetic acid varied between 15 and 18%. Lowering of the temperature, on reduction of the amount of KMnO_4 used, did not appreciably increase the yield.

3 (2' 5'-Dimethoxyphenyl) propane 1 2-diol The 'neutral' fraction of the above oxidation was concentrated to dryness, and the crystalline residue recrystallized from light petroleum (b p 120–130°). It had m p 77.5° (Found C, 62.2, H, 7.7. $\text{C}_{11}\text{H}_{14}\text{O}_4$ requires C, 62.2, H, 7.55%). Oxidation with periodic acid showed the substance to be a 1 2-glycol, 0.106 g of the diol was dissolved in a little warm water and added to 0.42M periodic acid (2 ml). The solution which became cloudy almost at once, due to the formation of the phenylacetaldehyde, was left for 1 5 hr. It was then extracted with ether, and the excess periodic acid titrated with 0.1N arsenite, 1.17 ml periodic acid had been used up, $\text{C}_{11}\text{H}_{14}\text{O}_4$ requires 1.19 ml. In another experiment the formation of formaldehyde was demonstrated by the isolation of the dimedone derivative.

Oxidation with performic acid A mixture of 30.5% (w/v) H_2O_2 (35 ml.) and formic acid (50 ml.) was allowed to stand at 18° for 0.5 hr. It was then added in three equal portions over 15 min to a stirred mixture of 1 4-dimethoxy 2 allylbenzene (53.4 g) and formic acid (90 ml.). After a lag period of 5–10 min the reaction started, the temperature was kept at 45–55° by cooling occasionally with ice water. After the exothermic reaction had subsided, the now homogeneous solution was kept at 50–60° for another 1.5 hr. The solution was concentrated under reduced pressure, and the residue refluxed with a slight excess of N NaOH in ethanol. The solution was neutralized, shaken with water and ether, and the ethereal solution dried. Removal of the ether gave a residue which was recrystallized three times from light petroleum (b p 120–130°). The diol was identified by its m p and titration with periodic acid. Yield was 20%. Swern *et al* (1946) have obtained much better yields with purely aliphatic olefins.

Oxidation with permanganate in water containing acetic acid

1 4 Dimethoxy 2-allylbenzene (20 g) was suspended in water (2 l.) containing acetic acid (46 ml), and the mixture stirred into an emulsion. KMnO_4 (57 g) dissolved in water (1 l.) was then added over 7 hr, whilst the temperature was kept at –1 to 0°. On working up the solution, as described by Hill & Short (1937), a small amount of 2 5 dimethoxyphenylacetic acid was obtained in addition to some diol.

Preparation of homogentisic acid from 2 phenyl 4 (2' 5' dimethoxybenzylidene) oxazolone

2 5 Dimethoxyphenylacetic acid The azlactone was converted to 2 5 dimethoxyphenylpyruvic acid as described by Gulland & Virden (1928). The crude keto acid (22.6 g) was oxidized with 8.4M H_2O_2 (12.5 ml) and 10N NaOH (12.75 ml) as described by these authors. At the end of the oxidation the alkaline solution was extracted with ether and then acidified with 36% (w/v) HCl (55 ml). The solution was then again extracted with ether, and the ethereal solution dried and concentrated. The solution, which was obtained on adding ethanol (150 ml) and conc H_2SO_4 (2.75 ml) to the remaining oil, was refluxed for 7 hr with exclusion of moisture. Most of the ethanol was then removed by distillation under reduced pressure, water (75 ml.) was added, and the two layers separated. The aqueous solution was extracted with ethyl acetate, and the combined oil and ethyl acetate extracts washed with NaHCO_3 . After drying and removal of the solvent, the oil was distilled at 20 mm. The first fraction (b p 102–104°) consisted of ethyl benzoate, whilst the second fraction which amounted to 16 g distilled at 170–175°. The latter material was saponified by boiling with 2.5N NaOH for 0.5 hr, extracted with ether and acidified. To the white precipitate was added enough water to dissolve most of the material on boiling, a small amount of oil was discarded, and the solution treated with charcoal. On filtration and cooling crystalline material was obtained which was recrystallized twice from water. M p was 125°. Yield after recrystallization was 70%.

Homogentisic acid The dimethoxy acid (7.8 g) was hydrolyzed by boiling with 120 ml. HBr (sp gr 1.48) for 5 hr. The solution was concentrated *in vacuo* under N_2 , and the residue taken up in hot water. This solution was again concentrated until crystals appeared. This solution containing some crystals was extracted three times with 10 vol. of ether. The ethereal solution, after drying, was concentrated until crystallization began and chloroform was added. The crystalline material was filtered off and identified as homogentisic acid. It had m p 147–148°, gave the typical reaction with FeCl_3 and reduced Ag^+ in acid solution. Yield was 80% of the theoretical.

Preparation of 2 5 dihydroxyphenylethylamine

3 (2' 5' Dimethoxyphenyl) propionic acid 3 (2' 5' Di hydroxyphenyl) acrylic acid (77 g) was dissolved in 0.5N NaOH (650 ml.) and hydrogenated with Raney nickel (10 g suspension) and hydrogen at 100 atm and at room temperature. The theoretical quantity of hydrogen was taken up after 1 hr. The catalyst was filtered off and the solution made acid to congo red. After cooling the product

was filtered off, it amounted to 74.5 g. After recrystallization from light petroleum (b.p. 120–130°) it had m.p. 66–67°. The material thus obtained was apparently mainly in the form of a hydrate, in spite of having been dried over P_2O_5 at 0.1 mm. (Found C, 59.0, H, 6.95. Calc. for $C_{11}H_{11}O_4$: C, 62.8, H, 6.6, calc. for $C_{11}H_{11}O_4 \cdot H_2O$: C, 58.0, H, 7.02%) Buck (1932) gives for the anhydrous acid m.p. 101°. The hydrated material was dried at 110° and at 0.1 mm over P_2O_5 for 3 hr. The oil was then crystallized from light petroleum (b.p. 120–130°). The crystalline material now had m.p. 100° in agreement with Buck.

Ethyl 3 (2' 5'-dimethoxyphenyl) propionate A mixture of 70 g. of the above acid, ethanol (100 ml.), benzene (150 ml.) and conc. H_2SO_4 (10 ml.) was refluxed for 10 hr. in a Soxhlet apparatus containing anhydrous $MgSO_4$ (50 g.) in the thimble. The cooled mixture was washed with water and the solvents removed. The ester distilled at 152–154° (1.5 mm). Yield was 90–95%.

3 (2' 5'-Dimethoxyphenyl) propionic hydrazide The above ester (38.4 g.) was refluxed for 6 hr. with 90% hydrazine hydrate (22 ml.) and addition of sufficient amyl alcohol to produce a homogeneous solution. The mixture was cooled, diluted with ether and the hydrazide extracted with 2N HCl. On neutralization with NaOH the hydrazide precipitated in crystalline form. After cooling it was filtered off and dried. On recrystallization from benzene or water m.p. of the hydrazide was 95–96° (Found C, 58.9, H, 7.1, N 12.6. $C_{11}H_{13}O_2N_2$ requires C, 58.9, H, 7.1, N, 12.5%).

2,5-Dimethoxyphenylethylamine To a solution of the hydrazide (34 g.) in 5N HCl (153 ml.), stirred at 0°, was added rapidly a solution of $NaNO_2$ (10.5 g.) in water (20 ml.). Ice was added to keep the temperature below 10°. The azide, which separated as a pale yellow oil, was extracted with cold benzene, and the benzene solution washed with ice-cold saturated NaCl. The solution was then dried at 0° over Na_2SO_4 and then over $CaCl_2$ and filtered. It was then warmed cautiously until evolution of N_2 began. After the reaction had abated, the solution was refluxed with exclusion of moisture for 20 min. Most of the solvent was distilled off, the residue was cooled and treated with cold HCl (200 ml. saturated at 0°). On warming, CO_2 was evolved. When the reaction appeared to be complete, most of the HCl was distilled off, leaving a crystalline residue of the hydrochloride. This had m.p. 142–143° after recrystallization from ethanol. The bulk of the hydrochloride was decomposed with excess NaOH, and the amine extracted with benzene. The benzene extract was dried and solvent removed. The amine distilled at 100° (0.5 mm). Yield was 80%.

2,5-Dihydroxyphenylethylamine hydrochloride All operations from now on were carried out, as far as possible, in an atmosphere of N_2 . The above amine was hydrolyzed by refluxing for 20 min. with 5 vol. of HI (sp. gr. 1.7) which had been freshly distilled over red phosphorus. Excess HI was removed under reduced pressure leaving a pale yellow oil which could be crystallized from acetone-ether. It had m.p. 173°. The bulk of the hydriodide was converted to the hydrochloride as follows: amine hydriodide (50 g.) was dissolved in water (15 ml.) containing a trace of SO_2 , the solution was shaken with freshly prepared $AgCl$ (30 g.) with addition of 5N HCl (0.5 ml.). The mixture of $AgCl$ and AgI was filtered off, washed with water, and the filtrate and washings concentrated to dryness. The oil which remained was dissolved in a small amount of ethanol and crystallized

by addition of dry ether. The hydrochloride was difficult to crystallize, owing to its great solubility and the ease with which it is oxidized, and it was found advisable to work with small amounts (2–3 g.). After several recrystallizations from ethanol-ether the amine hydrochloride had m.p. 169–170° (Found C, 50.8, H, 6.3, N, 7.1, Cl, 18.6. $C_8H_{11}O_2NCl$ requires C, 50.6, H, 6.3, N, 7.4, Cl, 18.8%). The amine formed a crystalline dilturate, however, this salt decomposed during recrystallization. The free amine which was prepared from the hydrochloride by addition of 1 equiv. of NaOH, crystallized well from water and had m.p. 128–130°. But attempts to recrystallize the base, in an atmosphere of N_2 , were unsuccessful owing to its instability. On keeping, the free base, sealed under nitrogen, decomposed within a few days to a black tar.

METABOLIC EXPERIMENTS

Methods

Feeding experiments and estimation of homogentisic acid in the urine were carried out as described by Neuberger (1947, 1948).

In experiments on man, H.A. was dissolved in pyrogen free water (twice glass distilled), and sufficient $NaHCO_3$ was added to neutralize about 90% of the acid. This solution was then passed through a Seitz filter, and the concentration of H.A. estimated iodometrically immediately before use. The final concentration was between 3 and 4%. The solution was then quickly injected into the antecubital vein, samples of 5 ml. blood were withdrawn before the injection and at intervals of 5 min., after the injection. H.A. was estimated by the micro iodometric and by the Folin method (Neuberger *et al.* 1947), and H.A. added to plasma before removal of the protein was used as standard.

Results

Toxicity of 2,5-dihydroxyphenylethylamine

The mice used for these experiments weighed between 20 and 25 g. 1 mg. of the amine hydrochloride (1% solution in water) given by vein to three mice had no effect, 2.5 mg. produced immediate death in two out of three animals, 5 mg. produced immediate death in three out of three mice. The surviving animals were observed for 3 days and appeared normal.

Feeding experiments on rats

Homogentisic acid This was given by mouth in doses of 50–500 mg./day to ten rats kept on normal diets, there were no reducing substances in the urine, either before or after hydrolysis with an equal amount of 1.5N-HCl at 100° for 1 hr.

2,5-Dihydroxyphenylethylamine hydrochloride This was given by mouth to seven rats in daily doses of 50–300 mg. There were no toxic effects, and no reducing substances could be demonstrated in the urine.

3 4-Dihydroxyphenylacetic acid The acid was given to five rats in daily doses of 25–100 mg. In all experiments the urine collected over the 24 hr following the administration reduced Ag^+ in alkaline solution, and gave a strong colour reaction with FeCl_3 . The reducing material was not isolated, but appears to have been an acid. It could be extracted into ether from an acid, but not from a neutral, aqueous solution and was most probably unchanged material. A rough semi-quantitative estimation with the aid of the FeCl_3 reaction indicated that about 75 mg of the 100 mg given were excreted.

Experiments on man

These experiments were done on two healthy males, weighing 63 and 69 kg respectively.

Ingestion by mouth Amounts of H.A. of 1–5 g dissolved in 10 vol of water were taken by mouth with and without an equivalent amount of NaHCO_3 . Urine was collected for 48 hr. Results were completely negative.

Injection by vein Altogether, seven experiments were carried out, four with 0.3 g H.A. and three with 1.0 g. The results with the two subjects were similar. With the smaller dose the excretion of H.A. in the urine began within 5 min and lasted for 15–25 min and most of the acid was eliminated in the first 10 min. The plasma level of H.A., 5 min after the injection, varied between 2.5 and 3.8 mg/100 ml to return to the initial value after about 15 min. With

the bigger dose, the urinary excretion of H.A. persisted for 28–40 min, the bulk again being eliminated in the first 10–15 min. The plasma level after 5 min varied between 8.0 and 10.5 mg/100 ml and returned to normal within 20–25 min. The total amount excreted was 28–35% of the injected dose with 0.3 g H.A. and 48–64% with 1.0 g H.A.

SUMMARY

1 The preparation of homogentisic acid by various methods has been studied. The most convenient synthesis found consisted of oxidation of 2,5-dimethoxyphenylpyruvic acid by hydrogen peroxide, followed by demethylation.

2 2,5-Dihydroxyphenylethylamine has been prepared by a Curtius degradation of the corresponding hydrazide.

3 This amine was found to be readily metabolized by the rat, and thus resembles other derivatives of hydroquinone. 3,4-Dihydroxyphenylacetic acid, on the other hand, was excreted largely unchanged as indicated by the reducing action of the urine.

4 Homogentisic acid, given by vein but not by mouth, to man, produced an alcaptonuria of short duration. It is concluded that the renal threshold for this acid is very low, even in the normal.

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The Steroids of Pregnant Mares' Urine

2 ISOLATION OF URANEDIOL SULPHATE*

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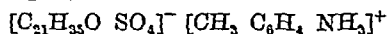
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The first paper of this series (Klyne, Schachter & Marran, 1948) describes a general method for the extraction of steroid conjugates from mares' urine and the isolation of an *allopregnenolone* sulphate. The present paper deals with the isolation from the 'water insoluble' fraction of the conjugates of a compound, *Y sulphate*, which appears to be the sulphate of the steroid 'uranediol', $C_{21}H_{36}O_2$, isolated from pregnant mares' urine by Marker, Rohrmann & Wittle (1938). Derivatives of 'urane', which may be a hydrocarbon isomeric with pregnane and *allopregnan*, have never been reported hitherto except by Marker and his co workers (Marker, Kamm, Crooks, Oakwood, Wittle & Lawson, 1938, Marker, Kamm, Oakwood, Wittle & Lawson, 1938, Marker, Lawson, Wittle & Crooks, 1938, Marker, Rohrmann & Wittle, 1938, Marker, Wittle & Oakwood, 1938, Marker & Rohrmann, 1939, Marker, 1941 *a, b*, 1944, see also Parke, Davis and Co 1940).

Subsequent to the isolation of *Y sulphate* Klyne & Paterson (1948) obtained from pregnant mares' urine a triol (*compound T*), apparently identical with 'uranetriol' (Marker, Kamm, Oakwood, Wittle & Lawson, 1938).

RESULTS

The '98% acetone solids, Fractions A and B', obtained by the general method of extraction (Klyne *et al* 1948) from two large batches of late pregnancy urine, consisted of the potassium salt of a new organic sulphate (*potassium Y sulphate*) in fairly pure form. The yields from the two batches were c 4 and 1 mg/l urine respectively. The potassium salt was transformed into *p toluidine Y sulphate*, the analyses and equivalent of which indicated that it was probably the salt of the monosulphate of an isomer of pregnanediol,



The *p toluidine* salt was hydrolyzed by hot acid to the free hydroxy compound, *Y*, analytical results for which agreed fairly well with those required for a saturated dihydroxy steroid, $C_{21}H_{36}O_2$. Compound

* Preliminary accounts of this work have been given to the Biochemical Society see Klyne (1946), Klyne & Paterson (1948).

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Y absorbed no hydrogen on shaking with Adams PtO_2 catalyst in glacial acetic acid for 2 hr, and gave negative tetranitromethane, Zimmermann and Millon reactions. It formed a precipitate with digitonin in 90% ethanol, indicating the probable presence of a 3(β) hydroxyl group. In concentrated H_2SO_4 , it gave an immediate orange red colour with a slight green fluorescence, and in the Liebermann-Burchardt test an immediate deep crimson colour.

Compound *Y* gave with acetic anhydride and pyridine at 15–20° *Y diacetate*, $C_{25}H_{40}O_4$, and with CrO_3 in glacial acetic acid at 15–20° an oxidation product *Y ketone*, $C_{21}H_{32}O_2$. The latter, on treatment with excess semicarbazide hydrochloride and sodium acetate at 15–20°, yielded a *monosemicarbazone*. *Y ketone* was recovered substantially unchanged after boiling with aqueous ethanolic HCl for 15 min.

Y ketone on Clemmensen reduction gave in c 50% yield a compound provisionally called *Y hydrocarbon*, which was saturated to tetranitromethane. A single elementary analysis on this compound gave results which differed considerably from those calculated for the expected formula (Found C, 84.4, H, 14.8. $C_{21}H_{36}$ requires C, 87.4, H, 12.6%). Lack of material prevented repetition of the Clemmensen reduction, and the author prefers to regard the nature of the product as doubtful, although Marker, Kamm, Oakwood, Wittle & Lawson (1938) gave for a product, obtained in the same way from 'uranetriol', analytical figures in good agreement with those required by $C_{21}H_{36}$.

EXPERIMENTAL

Methods

Melting points These were determined on the improvised Kofler hot-stage apparatus of Klyne & Rankellor (1947) and are corrected. Speed of heating at m.p., 3–4°/min. Limits of error, $\pm 2^\circ$.

Specific rotations See Klyne *et al* (1948).

Micro analyses These are by Dr J W Minnis unless otherwise stated.

'Usual working up' This expression indicates the following operations. A solution of the reaction product in an organic solvent (usually ether) was washed with aqueous Na_2CO_3 , aqueous H_2SO_4 and three times with water, it was then dried over anhydrous Na_2SO_4 , the latter filtered off, and the solution evaporated to dryness.

Isolation of *p* toluidine *Y* sulphate

The 'water insoluble' fraction (19.4 g) from 2701 late pregnancy urine was submitted to the '98% acetone' treatment (Klyne *et al* 1948) using 1×100 , 7×200 , 3×100 ml fractions. The acetone solutions yielded no solid on cooling, but on heating *without concentration* gave crystalline precipitates, Fraction *B* (1090 mg), which did not redissolve on cooling and were filtered off.

The 'water insoluble' fraction (9.9 g) from another batch of late pregnancy urine (530 l) was submitted to the '98% acetone' treatment (1×50 , 8×100 , 3×50 ml fractions). In this case the acetone solutions yielded on cooling to room temperature some dirty-white amorphous material (360 mg) from which no pure compound could be obtained. The filtrates from this material on standing at 0° gave a crisp white amorphous solid, Fraction *A* (360 mg). After this fraction had been removed by filtration the mother liquors were heated without concentration, but no Fraction *B* separated.

The above Fractions *A* and *B* (crude potassium *Y* sulphate), on treatment with *p* toluidine hydrochloride in aqueous solution as described for *Z* sulphate (Klyne *et al* 1948), yielded fairly pure *p* toluidine *Y* sulphate, which when recrystallized from water formed very fine white needles, *m p* 181–183° (decomp), $[\alpha]_D^{20} +16.1 \pm 0.3^\circ$ in ethanol (*c*, 3.0) (Found C, 66.6, 66.1, H, 9.1, 9.0, N, 2.8, S, 6.4, equivalent, 521, 522 $[C_{21}H_{25}OSO_4]^- [CH_3, C_6H_4, NH_3]^+$ requires C, 66.2, H, 8.9, N, 2.8, S, 6.3%, equivalent 507)

Compound *Y*

p Toluidine *Y* sulphate was hydrolyzed by hot *N* HCl as described for *Z* sulphate (Klyne *et al* 1948), except that 1 hr heating at 100° gave a better yield than 2 hr heating (88 as against 67%). Compound *Y* after repeated crystallization from ethanol (2 vol) water (1 vol.) formed needles, *m p* 211–213°, subliming at $180^\circ/0.06-0.1$ mm, $[\alpha]_D^{15} +3.7 \pm 0.8^\circ$ in ethanol (*c*, 1.8) (Found C, 78.3, 77.9, H, 11.4, 11.2 $C_{21}H_{35}O_2$ requires C, 78.7, H, 11.3%)

Compound *Y* (12 mg) was treated with acetic anhydride (2 ml) and pyridine (2 ml) for 12 hr at 15–20°. The product, *Y* diacetate, after the usual working up and recrystallization from aqueous methanol formed glistening plates, *m p* 159.5–160.5°, subliming at $190^\circ/0.1$ mm, $[\alpha]_D^{20} -30.4 \pm 0.7^\circ$ in chloroform (*c*, 1.4), $[\alpha]_D^{20} -29.6 \pm 1.1^\circ$ in ethanol (*c*, 1.0) (Found C, 74.0, 74.4, H, 10.2, 10.1 $C_{25}H_{40}O_4$ requires C, 74.2, H, 10.0%)

adding a few drops of ethanol and the mixture diluted with ether. The usual working up gave *Y* ketone, which after crystallization from ethanol/water (2 l) formed plates, *m p* 171–173°, subliming at $130^\circ/2 \times 10^{-4}$ mm, $[\alpha]_D^{20} -21.3 \pm 0.9^\circ$ in chloroform (*c*, 1.2) (Found C, 79.4, 79.4, H, 10.2, 10.0 $C_{21}H_{32}O_2$ requires C, 79.7, H, 10.2%)

Y ketone (4.5 mg) in ethanol (5 ml) was treated with semicarbazide hydrochloride (3.2 mg) and hydrated sodium acetate (4.5 mg) in water (0.5 ml), and allowed to stand at 15–20°. After 18 hr more semicarbazide hydrochloride (6.4 mg) and hydrated sodium acetate (10.0 mg) were added. After 3 days most of the ethanol was evaporated and the semicarbazone was precipitated with water, filtered, washed and dried. After recrystallization from ethanol it formed a white powder, *m p* 217–221° with browning, after softening 206–216° (Found N, 12.1 $C_{22}H_{35}O_2N_3$ (mono semicarbazone) requires N, 11.1 $C_{22}H_{35}O_2N_6$ (disemicarbazone) requires N, 19.5%)

Clemmensen reduction of *Y* ketone

Y ketone (6.0 mg) was dissolved in glacial acetic acid (2.5 ml) and conc HCl (2.5 ml). Amalgamated zinc wool (1 g) was added, and the mixture was refluxed for 7 hr, 1 ml of a mixture of glacial acetic acid and conc HCl (1:1) being added after each hour. The reaction mixture was diluted with hexane, and the usual working up gave a white solid (4.0 mg, *m p* not less than 90°). This material was chromatographed on Al_2O_3 (activity II, Brockmann & Schodder, 1941), pentane eluted 3 mg of a product which melted at 110–115° after softening from 103°, and recrystallized in long prisms on cooling to 109°. Recrystallization from acetone (*c* 1 ml) gave '*Y* hydrocarbon', glistening leaflets, *m p* 122–124° after softening at 116° (recrystallized partly on cooling to 123°) (Found C, 84.4, H, 14.8 $C_{21}H_{35}$ requires C, 87.4, H, 12.6%) (This analysis was carried out on 1.8 mg of substance in the Chemistry Department, University of Glasgow)

DISCUSSION

Comparison of the properties of compound *Y* and its derivatives with those reported by Marker, Rohrmann & Wittle (1938) for uranediol and its derivatives indicates that the two series of compounds are almost certainly identical (see Table 1), although the present author has been unable to obtain samples of Marker's materials for direct comparison.

Table 1 Comparison of compound *Y* and its derivatives with urane compounds

	<i>Y</i> series (<i>m p</i>)	Urane series, Marker (<i>m p</i> (uncorr.))
Diol, $C_{21}H_{34}(OH)_2$	211–213°	210°
Dioldiacetate, $C_{21}H_{34}(O CO CH_3)_2$	159.5–160.5°	160°
Ketone, $C_{21}H_{32}O_2$	171–173°	177.5°
Ketone semicarbazone, $C_{21}H_{32}O(N NH CO NH_2)$	217–221°	245°
Hydrocarbon, $C_{21}H_{36}$ (?)	122–124°	128°

Y ketone

Compound *Y* (24 mg), dissolved in glacial acetic acid (7.0 ml), was treated with a solution of CrO_3 (16 mg $\equiv 3$ atoms oxygen) in 98% acetic acid (1.2 ml) and allowed to stand for 18 hr. at 15–20°. Excess CrO_3 was reduced by

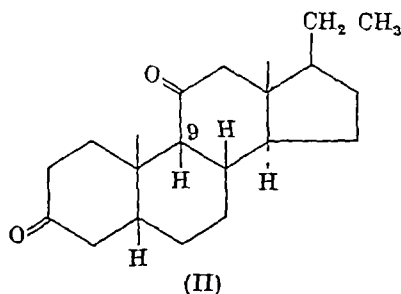
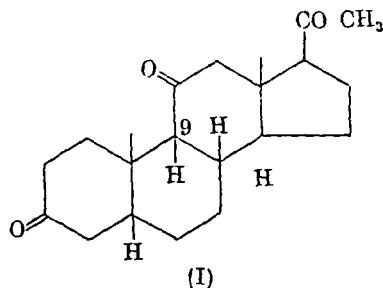
The only connexion between the urane series and compounds of known structure is a supposed transformation of uranetrione into pregnane-3,20-dione and uranediol reported by Marker, Kamm, Oakwood, Wittle & Lawson (1938) (see also Parke,

Davies and Co 1940, Marker, 1941a) The trans formation involved six stages, no intermediates were purified, and the final yield of pregnanedione was c 5%. The author feels that little weight can be given to such evidence and that the uranetriol, from which the uranetrione was originally made, may have been contaminated with a little pregnanediol.

Marker, Kamm, Oakwood, Wittle & Lawson (1938) suggested that urane was epimeric with pregnane at C₉, and that uranetrione was 9 *isopreg* 3 11 20 trione (I). They originally suggested that uranedione was the 3 20 diketone, but later Marker,

If 'urane' is a saturated C₂₁ steroid hydrocarbon it is certainly different from any known compound. Shoppee (1944) gave a list of C₂₁ hydrocarbons known at that date. His digmane is now known to be 5 *allo* 14 *iso* 17-*isopreg* 9 (Press & Reichstein, 1947), 5 *n* 14 *iso* 17 *isopreg* 9 has also been made (Meyer, 1947). The possibility that 'urane' is a C₂₀ or C₂₂ hydrocarbon should be considered, since analytical figures for compounds of these series do not differ greatly from those for the C₂₁ series.

The constitution of 'urane' will be investigated further when more material is available.



Rohrmann & Wittle (1938) formulated it as the 3 11 diketone (II). The partial transformation of uranetrione into pregnane 3 20 dione was thought to involve epimerization at C₉, at some stage (see also Marker, Wittle & Oakwood, 1938).

No evidence for or against a 9 *iso* structure for urane is available either from Marker's or from the present work, and the only evidence in favour of an 11 keto group in uranedione (Y ketone) and uranetrione is the inertness of one keto group in these compounds. Marker's suggested 11 keto-9 *iso* structure for these compounds is probably incorrect. Such a structure would be of *cis* α decalone type, and would be isomerized by heating with acid to the corresponding *trans* α decalone type (cf coprostane 3 6 dione, which gives with acid cholestane 3 6 dione, Butenandt, Schramm & Kudzusz, 1937), however, uranetrione (Marker, Kamm, Oakwood, Wittle & Lawson 1938) and Y ketone are stable to acid. The stability of these ketones to acid also excludes the possibility of a 14 *n* 17 *iso*-20 keto structure, since such compounds are isomerized by acid to the corresponding 14 *n* 17 *n* compounds (Butenandt, Schmidt-Thomé & Paul, 1939, Shoppee, 1940, Shoppee & Reichstein, 1940).

SUMMARY

1 A new organic sulphate has been isolated from pregnant mares' urine as its *p*-toluidine salt, which on acid hydrolysis yields compound Y, C₂₁H₃₆O₂, apparently identical with uranediol.

2 The reactions of compound Y have been studied.

3 Previous views on the structure of urane compounds are discussed.

The author is indebted to the Agricultural Research Council for a grant which defrayed the greater part of the expenses of this work, also to the Moray Fund and the Lewis Cameron Fund of the University of Edinburgh for other grants. He is grateful to N V Organon, Oss, Holland, for the gift of a large volume of mares' urine and facilities for working this up, also to British Drug Houses Ltd., the Ovaltine Research Laboratories and the Animal Diseases Research Institute, Moredun, Edinburgh, for gifts of urine. Without their willing co-operation this research could not have been carried out. He is also indebted to Prof G F Marrian, FRS, for constant help and encouragement, to Prof J W Cook, FRS, for one micro analysis and one hydrogenation carried out in his Department in the University of Glasgow, and to Mr A. Purdie for much valuable help in stable and laboratory.

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The Steroids of Pregnant Mares' Urine

3 ISOLATION OF ALLOPREGNAN-3(β)-OL-20-ONE SULPHATE*

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alloPregnan-3(β)-ol-20 one has been isolated from pregnant mares' urine after hydrolysis by Marker, Lawson, Wittle & Crooks (1938), by Heard & McKay (1939), and by Oppenauer (1941) The present paper describes the isolation of this compound as its sulphate

RESULTS

Pregnant mares' urine was extracted by the method described in Part 1 of this series (Klyne, Schachter & Marrian, 1948), and the crude conjugates obtained as potassium salts in certain '98 % acetone mother liquors' and '98 % acetone solids' were treated in aqueous solution with the hydrochlorides of various organic bases in an attempt to obtain crystalline derivatives The hydrochlorides used, which have been employed for the characterization of carboxylic and sulphonic acids and have also been found to give crystalline salts with simple organic sulphates (Barton & Young, 1943, Klyne, unpublished observations), were as follows *p* toluidine, benzylamine, β -phenylethylamine and cyclohexylamine hydrochlorides, piperazine dihydrochloride and *S*-benzyl ψ thiuronium chloride Piperazine dihydrochloride gave with the potassium salts of the crude conjugates a crystalline salt $[C_{21}H_{33}O SO_4]_2^- [C_6H_{12}N_2]^{++}$, *piperazine X sulphate*

This sulphate on acid hydrolysis gave a product (compound X), which, after purification as the

acetate, was shown to be identical with *allopregnan-3(β)-ol-20 one* Authentic *allopregnan-3(β)-ol-20 one* was converted into its sulphate (pyridine salt) by treatment with pyridine sulphur trioxide (cf Sobel & Spoerri, 1941), and the pyridine salt transformed into the piperazine salt This synthetic *piperazine allopregnanolone sulphate* and the *piperazine X sulphate* from mares' urine had identical melting points and mixed melting point However, analysis showed that the synthetic sulphate contained 2 molecules of water of crystallization even after drying *in vacuo* at 80° for 6 hr, while the mares' urine sulphate was anhydrous after drying for 1 hr in the same conditions Each sample had been crystallized from ethanol water, and no explanation of the difference in composition can be given

EXPERIMENTAL

Melting points These are corrected (see Klyne, 1948)

Micro-analyses These are by Dr J W Minnis

Specific rotations See Klyne *et al* (1948)

'Usual working up' See Klyne (1948)

Chromatograms These were carried out with Al_2O_3 , supplied by Peter Spence and Co Ltd., Widnes (Grade H, 100–200 mesh), the activity being determined according to Brookmann & Schodder (1941) The proportions used were as follows material to be fractionated, 1 g, Al_2O_3 , 30 g, eluent (each fraction), 100 ml In all cases the height of the Al_2O_3 column was 1–1.5 times its diameter The proportions of mixed solvents are given as % (v/v)

Isolation of X sulphate (*allopregnan 3(β)-ol 20 one sulphate*) as *piperazine salt* Late pregnancy urine (530 l) was extracted by the procedure previously described (Klyne *et al* 1948) and the 'water insoluble' fraction submitted to

* For a preliminary account of this work see Paterson & Klyne (1948)

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98% acetone extraction The acetone solutions from which potassium *Y* sulphate had been removed (see Klyne, 1948) were concentrated to a small volume and cooled, when amorphous solid, 'Fraction C', separated This was filtered off and the mother liquors evaporated to dryness, giving a light-brown solid (2.5 g) This material dissolved in the minimum volume of hot water was treated with piperazine dihydrochloride (1.2 g), dissolved in the minimum volume of water The light-brown precipitate which formed was recrystallized from water containing 10% (v/v) ethanol, giving 1.0 g of a product, m.p. 232–234° (decomp) further recrystallized three times from the same solvent, piperazine *X* sulphate formed small plates with m.p. constant at 230–241° (decomp) A further quantity of the same compound was similarly obtained from the amorphous '98% acetone solids, Fraction C', mentioned above

The salt was moderately soluble in water and rather more soluble in ethanol (Found (after drying *in vacuo* at 80° for 1 hr) C, 63.2, H, 8.9, N, 3.2, S, 7.7 [$C_{21}H_{33}O \cdot SO_4$], $[C_4H_{12}N_2]^{++}$ requires C, 62.6, H, 8.9, N, 3.2, S, 7.3%)

Compound *X* and *X* acetate Piperazine *X* sulphate (350 mg) was dissolved in hot water (500 ml) and ethanol (50 ml), and conc. HCl (50 ml) was added. The mixture was heated at 100° for 2 hr, cooled, and extracted with ether (1 × 300 ml, 3 × 150 ml), the usual working up gave

aqueous methanol, followed by another sublimation it had m.p. 137–140° *X* acetate gave no coloration with tetra-nitromethane, but gave a purple coloration in the Zimmermann ketosteroid test With digitonin in 90% (v/v) ethanol a ppt was slowly formed (cf. *allopregnan-3(β)-ol-20-one* acetate, Butenandt & Mamoli, 1935) (Found C, 76.7, 77.0, H, 9.8, 10.2 Calc for $C_{22}H_{35}O_3$ C, 76.6, H, 10.1%)

More *X* acetate was prepared from another sample of piperazine *X* sulphate, which had been obtained from the '98% acetone solids, Fraction C', from the same batch of urine The purification was similar to that described above, except that the crude free hydroxy compound was also submitted to a Girard separation

Hydrolysis of X acetate *X* acetate (18 mg) in methanol (2 ml) mixed with a solution of $KHCO_3$ (10 mg) in water (1 ml.) was refluxed for 1.5 hr After cooling the mixture was extracted with ether (100 ml) and worked up as usual The product, crystallized from benzene-hexane, gave compound *X* (8 mg), short rods, m.p. 190–194°, subliming at 140–150°/5 × 10⁻⁴ mm (Found C, 78.7, H, 10.6 Calc for $C_{21}H_{33}O_2$ C, 79.2, H, 10.8%) The properties of *X* and its acetate are compared with those of authentic *allopregnan-3(β)-ol-20-one* and its acetate (prepared by catalytic hydrogenation of *pregn-5-en-3(β)-ol-20-one* acetate (Plattner, Housser & Angliker, 1946)) in Table 1

Table 1 Comparison of compound *X* and *allopregnan-3(β)-ol-20-one*

Hydroxy compound	<i>X</i>	<i>allopregnanolone</i>
M.p.	192–195°	190–193°
Mixed m.p.		188–191°
$[\alpha]_D^{25}$ in chloroform (c, 1.0)	+89.7° ± 1.2°	+92.9° ± 0.7°
Acetate		
M.p.	137–140°	138–140°
Mixed m.p.		137–139°
$[\alpha]_D^{25}$ in chloroform (c, 1.0)	+72.1° ± 0.8°	+70.9° ± 0.5°
Piperazine salt of sulphate		
M.p. (decomp.)	239–241°	239–241°
Mixed m.p. (decomp.)		239–242°

Barton & Cox (1948) give the following values *allopregnanolone*, m.p. 194–195° (uncorr.), $[\alpha]_D^{25} + 95°$ in chloroform (c, 1) acetate, m.p. 143.5–144.5° (uncorr.), $[\alpha]_D^{25} + 77°$ in chloroform (c, 1)

181 mg product Chromatography of this material on Al_2O_3 (activity I–II) failed to yield any fraction of sharp m.p. The bulk of the material (130 mg), which was eluted with benzene-ether mixtures, was recombined, dissolved in 90% ethanol (5 ml) and treated with digitonin (500 mg) in 90% ethanol (5 ml) The precipitate was centrifuged, washed twice with 90% ethanol and dried (530 mg), it was then dissolved in dry pyridine (10 ml) and the solution diluted with dry ether (100 ml), yielding a ppt which was centrifuged and washed twice with ether, the ethereal solution and washings after thorough washing with dilute acid and the usual working up gave the digitonin precipitable fraction (105 mg) This fraction on acetylation (acetic anhydride (5 ml), pyridine (5 ml), 18 hr at 15–20°, usual working up) gave a colourless oil (120 mg), which was chromatographed (Al_2O_3 , activity II) Most of the material was eluted with hexane-benzene (90:10 and 80:20) mixtures These fractions, which had similar melting points, were combined and recrystallized from methanol-water (2:1, v/v) giving *X* acetate (24 mg), m.p. 124–130° After sublimation at 120°/4 × 10⁻⁴ mm, recrystallization from

Preparation of piperazine allopregnan-3(β)-ol-20-one sulphate *allopregnan-3(β)-ol-20-one* (320 mg) in dry benzene (14 ml) was mixed with pyridine sulphur trioxide reagent (320 mg) (Baumgarten, 1926, Baumgarten & Marggraff, 1931) and heated at 100° for 1.5 hr After cooling, light petroleum (b.p. 60–80°, 140 ml) was added and the mixture kept at 0° for 1 hr The white solid which separated was centrifuged and washed twice with small quantities of light petroleum The solid was then extracted six times with small quantities of warm $CHCl_3$, which, when filtered and evaporated, yielded a whitish solid This was taken up in $CHCl_3$, and hexane (approx. 1.5 vol.) added. On cooling at 0° pyridine *allopregnanolone* sulphate was obtained as small rods, 195 mg, m.p. 182–187° (decomp) This pyridine salt (17 mg) was dissolved in the minimum volume of water and treated with piperazine dihydrochloride (9 mg) in the minimum volume of water The precipitate which formed was recrystallized twice from water containing 10% (v/v) ethanol to give piperazine *allopregnan-3(β)-ol-20-one* sulphate (9 mg), m.p. 239–241° (decomp) (Found (after drying *in vacuo* at 80° for 6 hr) C, 60.3, 60.1, H, 8.5, 8.5,

N, 30, 30, S, 68 $[C_{21}H_{33}O SO_4^-]_2 [C_4H_{12}N_2^{++}]_2$, $2H_2O$ requires C, 60.1, H, 9.0, N, 3.1, S, 7.1 $[C_{21}H_{33}O SO_4^-]_2 [C_4H_{12}N_2]$ requires C, 62.6, H, 8.9, N, 3.2, S, 7.3% For comparison with piperazine X sulphate, see Table 1

SUMMARY

*allo*Pregnan 3(β) ol 20 one sulphate has been isolated from pregnant mares' urine and identified as its piperazine salt

We are indebted to the Agricultural Research Council for a grant which defrayed the greater part of the expenses of this work, also to the Moray Fund and the Lewis Cameron Fund of the University of Edinburgh for other grants. We are grateful to N V Organon, Oss, Holland, for the gift of a large volume of mares' urine and facilities for working this up. We are also indebted to Prof G F Marrian, F R S, for his continued interest and advice, and to N V Organon and to Prof T Reichstein (Basel) for gifts of pregn 5 en 3(β) ol 20 one acetate

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Carbon Dioxide Fixation in Animal Tissues

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It is now firmly established that carbon dioxide can be incorporated into organic compounds by animal tissues. Two primary reactions are known, viz

- (1) Pyruvate + $CO_2 \rightarrow$ oxaloacetate (Wood & Werkman, 1940)
- (2) α Ketoglutarate + $CO_2 \rightarrow$ oxalosuccinate (Ochoa, 1945)

Previous work on CO_2 fixation was concerned mainly with establishing its existence and studying the enzymic mechanisms involved, information on the rates of reactions (1) and (2) in animal tissues has remained scanty. The work reported in this paper was undertaken in order to collect data on the rates of these two reactions in various animal tissues and to assess their quantitative significance in metabolism.

METHODS

Owing to the instability and reactivity of the initial products the measurement of the rates of reactions (1) and (2) offers great experimental difficulties. Direct determination of oxaloacetate or of oxalosuccinate would not indicate the extent of reactions (1) or (2) because these compounds

undergo rapid secondary changes. Oxaloacetate yields fumarate, malate, succinate, citrate and α ketoglutarate, oxalosuccinate yields isocitrate, cisaconitate and citrate. These substances can arise under aerobic as well as an aerobic conditions.

Reaction (1) was examined in two ways. First, the sum of α ketoglutarate and succinate formed on anaerobic incubation with pyruvate and CO_2 was determined. Data published by Krebs, Eggleston, Kleinzeller & Smyth (1940), on the anaerobic products formed from oxaloacetate in various tissues, showed that the sum of succinate plus α ketoglutarate formed was fairly constant, amounting to 15–25% of the oxaloacetate utilized. The other products showed much greater variation. The sum of succinate plus α ketoglutarate formed, therefore, may be taken to represent approximately one fifth of the CO_2 fixed by reaction (1).

A second indirect method of studying the rate of reaction (1), applicable in O_2 only, is based on the fact that the oxidation of succinate is largely prevented by the addition of malonate. The interruption of the tricarboxylic acid cycle at the stage of succinate causes the bulk of the di- and tri-carboxylic acids to accumulate as succinate provided the rate of respiration is somewhat greater than that of the carboxylation of pyruvate. The amount of succinate found

on addition of malonate alone is thus an indicator of the total di and tri carboxylic acids in the tissue, and the increase found on addition of pyruvate may be ascribed to carboxylation.

For the study of reaction (2) it was decided to incubate tissues anaerobically with α ketoglutarate and to measure the formation of citrate. If one assumes that all the oxalo succinate formed through reaction (2) is reduced to iso citrate, then 89% of it would appear as citrate in the presence of aconitase. As it is possible that secondary reactions of citrate may occur, the rate of (2) may be greater than the amount of citrate formed indicates. On the other hand, citrate can be formed under some conditions by other mechanisms, but these were probably quantitatively insignificant except when oxaloacetate was present. Anaerobic conditions were chosen in order to exclude the formation of citrate by the oxidative reactions of the tricarboxylic acid cycle.

EXPERIMENTAL

The experiments were carried out on minced or homogenized material. Pigeon, rat and guinea pig brains were homogenized in a stainless steel homogenizer similar to the glass apparatus of Potter & Elvehjem (1936). Pigeon liver was minced in the Fischer apparatus (Fischer, 1925), other tissues in the Latapie mill. The minced tissue was suspended in saline medium in the proportion 1 part of tissue plus 6.5 parts of medium, unless otherwise stated. The following media were used.

(1) Phosphate saline pH 7.4 (Krebs & Eggleston, 1940a), with N_2 or O_2 .

(2) Phosphate saline bicarbonate, prepared by adding 16 ml. of 1.3% $NaHCO_3$ to 100 ml. of medium (1). Gas 5% CO_2 in N_2 or O_2 . The final bicarbonate concentration was 0.0214M, and when equilibrated with the gas mixture the pH of this medium was approximately 7.4.

To prepare a boiled yeast extract a suspension of 1 g. of dried baker's yeast in 10 ml. of water was placed in a boiling water bath for 10 min. and then filtered. Boiled muscle extract was similarly prepared from a mixture of 1 part of minced pigeon breast muscle and 1 part of phosphate saline.

Tissue suspension (3 ml.) was pipetted into the main compartment of a Warburg cup. Further additions (substrates, inhibitors, buffer or water) amounting to 1 ml. were placed in the side arm, so that upon mixing the final suspension contained 1 part of tissue in 10 parts of suspension. In anaerobic experiments a stick of yellow phosphorus was placed in the centre well. The contents of the side arm were mixed with those of the main compartment immediately before placing the manometers in the water bath at 40°. At the end of the incubation period the manometers were removed from the bath, the cups detached, placed in iced water, and 1 ml. of 2N HCl was added to the suspensions to stop the reaction.

For the determination of the sum of succinate and α ketoglutarate, the acidified tissue suspension was deproteinized with tungstic acid. Succinate and the sum of succinate plus α ketoglutarate were determined by the procedure of Krebs & Eggleston (1945) in the earlier experiments. Later, when it was realized that glutamate interfered, a modified method (Krebs & Eggleston, 1948) was used. Glutamic acid was determined according to Gale (1945). It represented the sum of glutamic acid plus glutamine. For

the determination of citrate the contents of the cup were washed with water into a 50 ml. measuring cylinder, diluted to 45 ml. and mixed with 5 ml. of 20% metaphosphoric acid. A measured part of the clear filtrate of this suspension was used for the citrate determinations. Citric acid was determined colorimetrically in the manner described by Krebs & Eggleston (1944) using a Spekker absorptiometer and a no. 6 filter.

For purposes of calculation the dry weight was taken to be 30% of the wet weight in the case of liver and 20% for other tissues.

RESULTS

Anaerobic formation of succinate and α ketoglutarate in presence of pyruvate

Various tissues of the pigeon (liver, brain), rat (liver, kidney cortex, skeletal muscle), guinea pig (liver, brain), and sheep (liver, kidney cortex, heart, brain, spleen, pancreas and lung) were examined. Only in pigeon liver, which is already known to convert pyruvate into succinate and α ketoglutarate, was an increased yield of these substances observed. As the experiments on other tissues gave essentially negative results they will only be summarily reported. In the first experiments all tissues appeared to contain initially some 'succinate plus α ketoglutarate' (25–150 mg/100 g wet wt., expressed as succinic acid). On anaerobic incubation without addition of pyruvate (40 min. at 40°), these dicarboxylic acids increased by 20–60 mg/100 g in all tissues tested save rat muscle and sheep lung. Addition of pyruvate (0.02M) did not result in any further increase in these dicarboxylic acids, except in pigeon liver.

At this stage of the investigation it was realized that glutamic acid interfered with the determination of α ketoglutarate and succinate by also yielding succinate on treatment with permanganate, and some of the above experiments were then repeated with the modified method avoiding this source of error (Krebs & Eggleston, 1948). With this method 400 mg. of fresh avian or mammalian tissue yielded no detectable amounts of succinate or α ketoglutarate (i.e. less than 0.05 mg.) and Gale's (1945) method showed that they contained 0.4–1.3 mg. of glutamic acid. Further, on anaerobic incubation of mammalian tissues at 40° (pH 7.4, no substrate added, 40 min.) the amount of glutamic acid increased by 50% or more, possibly as a result of hydrolysis of proteins or peptides, whilst little or no succinic acid (< 0.05 mg.) and no α -ketoglutaric acid was formed. It must, therefore, be concluded that the succinic acid found in the earlier experiments had arisen from glutamic acid by oxidation with permanganate.

The above results do not contradict those of Moyle (1924) who found that succinic acid was present in muscle and increased on anaerobic incubation, for the amounts she reported would not be

measurable by the procedure employed in the present work

In confirmation of previous work it was found that succinate and α -ketoglutarate were formed by pigeon liver on anaerobic incubation in the absence of pyruvate, and that addition of pyruvate (0.02–0.04M) increased the yields. In contrast to mammalian tissues, no change occurred in the initial glutamic acid concentration of pigeon liver on anaerobic incubation without pyruvate. In Table 1

Table 1 *Anaerobic synthesis of succinate plus α -ketoglutarate in pigeon liver*

(400 mg fresh liver in 4 ml suspension. $Q_{\text{succinate}}$ refers to the increase in the sum of succinate and α -ketoglutarate during incubation. The initial value of the sum was 76 $\mu\text{l}/400 \text{ mg}$.)

Gas	Incubation time (min)	$Q_{\text{succinate}}$	
		No pyruvate	0.04M pyruvate
5% CO_2 in N_2	20	2.1	3.8
	40	1.4	2.1
5% CO_2 in O_2	20	2.5	6.3
	40	1.8	5.0

the rates of formation of succinate plus α -ketoglutarate by pigeon liver are given in terms of the metabolic quotient Q ($\mu\text{l}/\text{mg}$ dry wt/hr). These rates are comparable in magnitude to those reported earlier from this laboratory (Evans, 1940; Krebs & Eggleston, 1940b). On the assumption that anaerobically 20% of the total oxaloacetate formed by reaction (1) was converted to succinate plus α -ketoglutarate, the initial rate of reaction (1) was 19 in the presence of pyruvate and in N_2 . In all the other tissues examined, where no formation of succinate plus α -ketoglutarate was detectable on addition of pyruvate (i.e. less than 0.05 mg was present in 400 mg wet tissue after 40 min incubation), the rate of reaction (1) in terms of $Q_{\text{oxaloacetate}}$ must have been below 1.

The malonate technique was applied to the same tissues. Addition of 0.02M-pyruvate to tissues suspended in a saline medium containing 0.01M-malonate did not increase the yield of succinate or of α -ketoglutarate beyond that obtained with malonate alone except in pigeon liver and sheep kidney cortex. In the latter tissue $Q_{\text{succinate}}$ varied between 0 and 1.0 (average $Q = 0.6$ for five experiments) with malonate alone and was increased by addition of pyruvate to values between 0.6 and 3.0 (average $Q = 1.7$ for five experiments). In pigeon liver Evans (1940) found a $Q_{\text{succinate}}$ of 2.7 in the presence of 0.025M-malonate and of 8.7 with malonate and 0.03M-pyruvate. With the revised technique for determination of succinate and α -ketoglutarate, similar values were obtained. $Q_{\text{succinate}}^{\text{O}_2}$ was 4.7 with malonate and 7.4 with malonate and pyruvate (10 min incubation). With regard to

the interpretation of these figures it is relevant that the aerobic conversion of oxaloacetate into succinate requires three oxygen atoms. As the Q_{O_2} of pigeon liver in the presence of pyruvate is about 18 the maximum value for $Q_{\text{succinate}}^{\text{O}_2}$ to be expected by aerobic reactions is about 12. Additional succinate and α -ketoglutarate, as already discussed, can be formed by anaerobic reactions, but it is clear that if the rate of carboxylation of pyruvate, in terms of $Q_{\text{oxaloacetate}}$, is about 12 or more, only part of the oxaloacetate will be converted into succinate. The yield of succinate in the malonate experiments is thus not incompatible with a $Q_{\text{oxaloacetate}}$ of 19 in pigeon liver, computed from other experiments.

Anaerobic formation of citrate in presence of α -ketoglutarate

(a) Pigeon liver

1 *Citrate synthesis* Table 2 shows the amounts of citrate formed by minced pigeon liver suspensions upon anaerobic incubation with various substrates.

Table 2 *Anaerobic citrate synthesis from α -ketoglutarate and other substrates in pigeon liver*

(400 mg fresh tissue in 4 ml suspension. Incubation period 12 min. Phosphate saline bicarbonate (0.02M) medium, 5% CO_2 in N_2 .)

Exp	Additions	Citrate formed (μl)
1	None	~0
	0.04M- α -Ketoglutarate	91
2	None	~0
	0.04M α -Ketoglutarate	92
	0.02M Pyruvate	23
3	0.02M- α -Ketoglutarate	72
	0.02M Oxaloacetate	124
	0.02M Oxaloacetate,	206
	0.02M pyruvate	

Pyruvate and oxaloacetate both alone and in combination yielded citrate, confirming the results of Krebs & Eggleston (1940b) and of Evans (1940). Amounts of citrate of similar magnitude were also formed on addition of α -ketoglutarate. In ten separate experiments Q_{citrate} varied from 2.4 to 4.1 (average 3.8) when the incubation period was 12 min. Other substrates such as L-lactate, L-glutamate, fumarate, succinate, acetoacetate and β -hydroxybutyrate (all 0.02M final concentration) did not form measurable amounts of citrate, i.e. less than 20 $\mu\text{l}/120 \text{ mg}$ dry liver.

2 *Time course of citrate synthesis from α -ketoglutarate* Table 3 demonstrates the time course of the citrate formation from added α -ketoglutarate. The initial rate was very rapid ($Q_{\text{citrate}} = 30$), but the reaction quickly came to a standstill, the maximum yield of citrate was obtained within 5 min.

Table 3 *Time course of citrate synthesis from α ketoglutarate in pigeon liver*(0.02M- α Ketoglutarate Other conditions as in Table 2)

Time (min)	Citrate formed (μ l)	Q_{citrate}
1	60	30
2	82	—
4	95	—
12	98	—

3 *Effect of α ketoglutarate concentration* The amount of citrate formed increased when the concentration of α ketoglutarate was raised from 0.005 to 0.02M (Table 4). Increasing the concentration beyond 0.02M did not raise the yield of citrate.

Table 4 *Effect of α ketoglutarate concentration on citrate synthesis in pigeon liver*

(Conditions as in Table 2)

α Ketoglutarate (M)	Citrate formed (μ l)
0.005	33
0.01	57
0.02	92
0.04	94

Table 5 *Effect of bicarbonate (or carbon dioxide) concentration on citrate synthesis in pigeon liver*(Except for CO_2 and bicarbonate concentration, conditions as in Table 2)

Gas (% CO_2 in N_2)	Bicarbonate (M)	pH (calc)	Citrate formed with substrates (μ l)	
			0.02M- α Keto glutarate	0.02M- α Keto glutarate + 0.02M fumarate
0	0.0019	7.40	26	41
2.14	0.0105	7.42	37	113
4.52	0.0217	7.41	55	143
10	0.0591	7.45	87	162

4 *Effect of bicarbonate and carbon dioxide concentration* In the following experiments the concentrations of bicarbonate and of carbon dioxide were varied in about the same proportions so that the pH remained approximately constant. Various amounts of 1.3% bicarbonate solution were added to a suspension of minced liver in phosphate saline medium and the gas spaces of the vessels were filled with a corresponding mixture of carbon dioxide in nitrogen. The actual bicarbonate concentration of the final tissue suspension was determined experimentally in each instance on a duplicate sample. The amount of citrate formed rose as the concentration of bicarbonate increased from 0.002 to 0.06M (Table 5). The optimum concentration of bicarbonate appeared to be rather higher than 0.02M, the concentration employed routinely in other experiments because this is the approximate concentration of bicarbonate

in the blood plasma. Three experiments performed in phosphate saline medium and in an atmosphere of nitrogen without carbon dioxide with alkali in the centre well to absorb carbon dioxide, yielded small, just measurable, amounts of citrate in each case ($30 \pm 5 \mu$ l). It was impossible to exclude carbon dioxide rigorously under these conditions, because of its constant production from α ketoglutarate by the tissue suspension.

5 *Effect of pH of medium* The pH was varied by changing the carbon dioxide content of the gas mixtures. Thus, in addition to changes in pH, the concentration of free carbon dioxide varied, while that of bicarbonate remained constant. The data of Table 6 show that citrate formation increased when pH fell from 7.73 to 6.95. It was the same at pH 7.73 and 8.04.

6 *Effect of various substrates* Representative effects of various substrates on the anaerobic citrate formation from α ketoglutarate are shown in Table 7. Acetoacetate, β -hydroxybutyrate, and succinate (each in 0.02M concentration) inhibited citrate formation about 30%, 0.02M-L lactate caused an inhibition of 60% and 0.02M-pyruvate was without effect.

Table 6 *Effect of pH on citrate synthesis in pigeon liver*

(0.02M Bicarbonate Other conditions as in Table 2)

Gas (% CO_2 in N_2)	pH (calc)	Citrate formed (μ l)
1.05	8.04	61
2.14	7.73	60
4.52	7.40	98
12.8	6.95	112

Ammonium chloride (0.02M) completely abolished citrate formation. α Ketoglutarate and ammonia are known to form glutamic acid under the conditions of the present experiments and this reaction can be coupled with the conversion of citrate into α -ketoglutarate (Adler, von Euler, Gunther & Plass, 1939; Krebs, Eggleston & Hems, 1947). The effect

Table 7 *Effect of various substrates on citrate synthesis in pigeon liver*

(0.04M- α Ketoglutarate present in each instance Other conditions as in Table 2 Value in presence of β hydroxy butyrate has been corrected for interference)

Exp	Additions	Citrate formed (μ l.)
1	None	91
	0.02M L Lactate	36
	0.02M β Hydroxybutyrate	67
	0.02M NH_4Cl	~ 0
2	None	92
	0.02M Pyruvate	94
	0.02M Acetoacetate	64
	0.02M Succinate	66
	0.02M L-Glutamate	109

of ammonium chloride on the yield of citrate is probably connected with this dismutation. L Glutamate had no appreciable effect on citrate formation. The small increase shown in Table 7 was not found regularly.

Fumarate (0.02M) caused an increased yield of citrate in the presence of α -ketoglutarate (Tables 5 and 8). The increase varied from 20 to 100% in different livers. As with α -ketoglutarate alone the maximum yield of citrate in the presence of both α -ketoglutarate and fumarate was reached within 12 min. The effect of fumarate depended on the bicarbonate and carbon dioxide concentrations (Table 5) and was greatest at suboptimal concentrations. Fumarate alone did not yield citrate. Malate was not examined since it may be taken for granted that it behaves like fumarate.

Oxaloacetate alone, as is well known, gives rise to citrate and in a short term (12 min) experiment (Table 8, Exp 1) the effects of oxaloacetate and

α ketoglutarate were roughly additive. In an experiment where the period of incubation was 30 min (Table 8, Exp 2) oxaloacetate and α -ketoglutarate together gave more citrate than could be accounted for by additive action only.

As Ochoa's (1945) mechanism of citrate formation involves the reduction of oxalosuccinate to isocitrate, it was thought that addition of a hydrogen donor might accelerate the formation of citrate. Hypo sulphite which is known to regenerate the reduced form of the coenzymes (Warburg, Christian & Griese, 1935) was, therefore, added (0.036M), but it caused a considerable inhibition (Table 8, Exp 1) which was not affected by simultaneous addition of lactate.

A detailed study of the inhibition by L lactate showed that the inhibition was greater the higher the lactate concentration (Table 9, Exp 1), and that it was of the same order in the presence of added fumarate or oxaloacetate as with α ketoglutarate alone. Lactate also inhibited citrate formation from oxaloacetate alone, and from oxaloacetate plus pyruvate (Table 9, Exp 2). Pyruvate (0.02M) did not affect lactate inhibition. In some experiments L glutamate (0.02M) partly or wholly reversed the inhibition by lactate (Table 9, Exps 2 and 3). The anaerobic or oxidative removal of citrate was not affected by 0.04M L-lactate.

7 Aerobic citrate synthesis Aerobically the yields of citrate in the presence of α ketoglutarate were about 40% higher than anaerobically (Table 10). The effects of various substances in the presence of oxygen were similar to those observed in anaerobic experiments. Since the interpretation of aerobic experiments is complicated by the fact that citrate may arise by other reactions (i.e. through the tricarboxylic acid cycle) these experiments were not pursued.

Table 8 *The effect of oxaloacetate, fumarate, lactate and hyposulphite ($\text{Na}_2\text{S}_2\text{O}_4$) on citrate synthesis in pigeon liver*

(Incubation period 12 min for Exp 1 and 30 min for Exp 2 Other conditions as in Table 2)

Exp	Additions	Citrate formed (μ l.)
1	0.02M- α Ketoglutarate	61
	0.02M α Ketoglutarate, 0.02M oxaloacetate	165
	0.02M Oxaloacetate	121
	0.036M Hyposulphite	~ 0
	0.036M Hyposulphite, 0.02M- α ketoglutarate	29
	0.036M Hyposulphite, 0.02M α ketoglutarate, 0.02M lactate	31
2	0.02M- α Ketoglutarate	98
	0.02M- α Ketoglutarate, 0.02M oxaloacetate	182
	0.02M Oxaloacetate	36
	0.02M Oxaloacetate, 0.02M fumarate	88
	0.02M Oxaloacetate, 0.02M fumarate, 0.02M α ketoglutarate	243
	0.02M- α Ketoglutarate, 0.02M fumarate	172
	0.02M α Ketoglutarate, 0.02M fumarate, 0.02M lactate	53
	0.02M α Ketoglutarate, 0.02M lactate	31
	0.02M Oxaloacetate, 0.02M lactate	~ 0
	0.02M α Ketoglutarate, 0.02M oxaloacetate, 0.02M lactate	60

Table 9 *The effect of lactate on citrate synthesis under various conditions in pigeon liver*

(Conditions as in Table 2)

Exp	Additions	Citrate formed (μ l)
1	0.02M- α Ketoglutarate	98
	0.02M α Ketoglutarate, 0.02M fumarate	151
	0.02M α Ketoglutarate, 0.005M lactate	69
	0.02M α Ketoglutarate, 0.005M lactate, 0.02M fumarate	83
	0.02M α Ketoglutarate, 0.01M lactate	50
	0.02M α Ketoglutarate, 0.01M lactate, 0.01M fumarate	80
	0.02M α Ketoglutarate, 0.04M lactate	36
2	0.02M α Ketoglutarate	72
	0.02M α Ketoglutarate, 0.02M pyruvate	71
	0.02M α Ketoglutarate, 0.02M pyruvate, 0.02M lactate	39
	0.02M α Ketoglutarate, 0.02M lactate, 0.02M L glutamate	76
	0.02M Oxaloacetate	124
	0.02M Oxaloacetate, 0.02M lactate	60
	0.02M Oxaloacetate, 0.02M pyruvate	206
3	0.02M Oxaloacetate, 0.02M pyruvate, 0.02M lactate	84
	0.02M- α Ketoglutarate	100
	0.02M α Ketoglutarate, 0.01M lactate	49
	0.02M α Ketoglutarate, 0.02M L-glutamate	112
	0.02M- α Ketoglutarate, 0.02M L-glutamate, 0.01M lactate	81

Table 10 *Effect of oxygen on citrate synthesis in pigeon liver*(5% CO₂ in O₂ Other conditions as in Table 2)

Additions	Citrate formed (μ l)
0.04M- α Ketoglutarate	164
0.02M α Ketoglutarate	142
0.02M- α Ketoglutarate, 0.02M fumarate	192
0.02M Fumarate	39
0.02M α -Ketoglutarate, 0.02M pyruvate	133
0.02M- α Ketoglutarate, 0.02M L lactate	69
0.02M α Ketoglutarate, 0.01M acetoacetate	109
0.02M- α Ketoglutarate, 0.01M β hydroxybutyrate	117

8 *Effect of inhibitors* The addition of 0.02M malonate caused 32% inhibition of citrate formation in nitrogen and 65% inhibition in oxygen (Table 11). The inhibition in oxygen was partly reversed by

addition of fumarate. Sodium fluoride, 0.02M, inhibited citrate formation in nitrogen by 47% (Table 11). Adenosinetriphosphate (0.002M), cozymase (5 μ g/ml) or 0.001M-MnCl₂ had no effect on citrate formation from α ketoglutarate or on the inhibition by lactate. Thus none of these substances were limiting factors under the conditions of these experiments.

(b) Other tissues

The formation of citrate in the presence of α ketoglutarate was examined in other tissues. It occurred to a considerable extent in pigeon breast muscle, guinea pig and rat liver, and to a less extent in sheep liver, heart and kidney cortex (Table 12). No citrate formation was detected in guinea pig, rat and sheep brain, sheep spleen, and rat and sheep testis, though the latter tissue contained large amounts of citrate (see Schersten, 1936, Dickens, 1941).

Table 11 *The effect of malonate and fluoride on citrate synthesis in pigeon liver*

(Incubation period 12 min in Exps 1-3, 30 min in Exp 4 Other conditions as in Table 2)

Exp	Gas	Additions	Citrate formed (μ l)	Inhibition (%)
1	5% CO ₂ in O ₂	0.04M- α Ketoglutarate	136	—
		0.04M- α Ketoglutarate, 0.02M malonate	42	69
		0.04M- α Ketoglutarate, 0.002M malonate	88	37
2	5% CO ₂ in O ₂	0.02M- α Ketoglutarate	142	—
		0.02M- α Ketoglutarate, 0.02M fumarate	192	+35
		0.02M- α Ketoglutarate, 0.02M malonate	50	65
		0.02M α Ketoglutarate, 0.02M malonate, 0.02M fumarate	123	36
3	5% CO ₂ in N ₂	0.02M- α Ketoglutarate	61	—
		0.02M α Ketoglutarate, 0.02M malonate	41	32
		0.02M- α Ketoglutarate, 0.02M Na fluoride	32	47
4	5% CO ₂ in N ₂	0.02M- α Ketoglutarate	158	—
		0.02M- α Ketoglutarate, 0.02M malonate	110	31
		0.02M- α Ketoglutarate, 0.005M malonate	135	14

Table 12 *Citrate synthesis by various tissues in the presence of α -ketoglutarate*

(400 mg fresh tissue in 4 ml. suspension except with sheep testis (800 mg) and rat testis (250 mg) Incubation period 20 min except with pigeon breast muscle (12 min) 5% CO_2 in N_2 or O_2)

Tissue	Gas	Additions	Citrate formed (μl)
Pigeon-breast muscle	N_2	0.02M α Ketoglutarate	47
	O_2	0.02M α Ketoglutarate	30
		None	~0
Guinea-pig liver	N_2	0.04M α Ketoglutarate	69
		0.04M α Ketoglutarate, 0.02M fumarate	123
	O_2	0.04M α Ketoglutarate	53
		0.04M α Ketoglutarate, 0.02M fumarate	180
		None	~0
Rat liver	N_2	0.04M α Ketoglutarate	48
		0.04M α Ketoglutarate, 0.02M fumarate	66
		0.04M α Ketoglutarate, 0.02M lactate	~0
		None	~0
	O_2	0.04M α Ketoglutarate	69
		0.04M α Ketoglutarate, 0.02M fumarate	133
Sheep liver	N_2	0.04M α Ketoglutarate	25
		0.04M α Ketoglutarate, 0.02M fumarate	32
		0.04M α Ketoglutarate, 0.02M lactate	~0
Sheep heart	N_2	0.04M α Ketoglutarate	35
		0.04M α Ketoglutarate, 0.02M fumarate	55
		0.04M α Ketoglutarate, 0.02M L glutamate	26
Sheep kidney	O_2	0.04M α Ketoglutarate	25
		0.04M α Ketoglutarate, 0.02M fumarate	38
	N_2	0.04M α Ketoglutarate, 0.02M fumarate	26
Sheep testis	N_2	None	24
		0.04M α Ketoglutarate	25
		0.04M α Ketoglutarate, 0.02M fumarate	37
Rat testis	N_2	None	32
		0.04M α Ketoglutarate	41
		0.04M α Ketoglutarate, 0.02M fumarate	39

DISCUSSION

Rate of carbon dioxide fixation by pyruvate in minced and homogenized animal tissues In pigeon liver the computed rate for the carboxylation of pyruvate ($Q_{\text{oxalosuccinate}} = 19$) was of the same order of magnitude as the rate of respiration. In sheep-kidney cortex the rate as determined by the malonate technique was, on average, 1.7. In all other tissues examined (rat liver, kidney cortex and muscle, pigeon brain, guinea pig liver and brain, and sheep liver, heart, brain, spleen, pancreas, and lung) the rate of carboxylation must have been less than 1 since no formation of succinate or α ketoglutarate could be measured by the methods used. It should be emphasized that the non-detection of the reaction only indicates that the rate was below 1 under the given experimental conditions. Experiments published by Solomon, Vennesland, Klemperer, Buchanan & Hastings (1941), Wood, Lifson & Lorber (1945) and Anfinsen, Beloff, Hastings & Solomon (1947), in fact, make it very probable that the carboxylation of pyruvate occurs in mammalian

liver. It can be calculated from the data given by Buchanan, Hastings & Nesbitt (1942) that the rate at which carbon dioxide was incorporated in glycogen in rabbit liver slices under their experimental conditions was, in terms of $Q_{\text{CO}_2}^{\text{glycogen}}$, between 0.14 and 0.48 in four experiments (average 0.26). As aspartic and glutamic acids containing carbon dioxide were synthesized at the same time (Anfinsen *et al.* 1947) these figures are the minimum rates. They are compatible with the conclusion reached from the present experiments that the rate of the carboxylation of pyruvate is below 1 in mammalian liver.

Mechanism of citrate formation from α ketoglutarate The dependence of citrate formation upon the concentration of α -ketoglutarate and of bicarbonate and carbon dioxide in these experiments is in accordance with the conception, already firmly established by Ochoa (1945), Grisolia & Vennesland (1947), and Floyd, Medes & Weinhouse (1947) that the primary step is the carboxylation of α ketoglutarate and formation of oxalosuccinate, followed by its reduction to isocitrate and rearrangement to citrate. These three stages involve at least three enzyme

systems oxalosuccinic carboxylase, isocitric dehydrogenase and aconitase. The stimulating effect of fumarate, and the inhibitory action of lactate which can be partially reversed by glutamate, are presumably connected with the second stage, but no explanation for the cause of the effects can be offered. It is of interest that lactate also inhibits the formation of citrate from oxaloacetate and pyruvate which is an oxidative process.

Rate of carbon dioxide fixation by α ketoglutarate
The formation of citrate on addition of α -ketoglutarate was demonstrable in pigeon liver, pigeon breast muscle, guinea pig, rat and sheep liver, sheep heart and sheep kidney cortex, whilst negative findings were obtained with guinea pig, rat and sheep brain, sheep spleen and rat and sheep testis. In pigeon liver the rate was very high immediately after addition of the α ketoglutarate but fell to zero within a few minutes. Q_{citrate} was 30 during the first minute. The total amounts of citrate formed were independent of the concentration of α -ketoglutarate at the higher concentrations, and the stoppage of the synthesis, therefore, cannot be ascribed to a simple equilibrium effect.

In view of the rapid change of rate, Q values calculated from data referring to experimental periods of more than a few minutes indicate only a minimum value for the initial velocity. In the experiments given in Table 12 the period of incubation was 12 or 20 min and the average Q values for the period of incubation reached 2.9 in pigeon-breast muscle, 4.6 in guinea pig liver, 3.3 in rat liver, 0.8 in sheep liver, and 2.1 in sheep heart. The initial Q values were in all probability much higher.

Significance of the carbon dioxide fixation by α keto glutarate
Grisolia & Vennesland (1947) have already pointed out that the conversion of citrate into α ketoglutarate is the only metabolic process which citrate, once formed, is definitely known to undergo. Hence it would appear that the carboxylation of α ketoglutarate cannot result in the incorporation of assimilated carbon in any compounds other than the four tricarboxylic acids. These acids can be readily formed in animal tissues through the oxidative reactions of the tricarboxylic acid cycle, and as long as no further reactions of citrate are known, it is difficult to visualize the specific biological significance of the carboxylation of α ketoglutarate. It has

been suggested (Dr H. Mellin, personal communication) that the formation of citrate from α -ketoglutarate, which is an endergonic reaction, might serve as a temporary storage of energy. If the idea of Lipton & Barron (1946) that citrate might undergo fission to oxaloacetate and acetate should prove correct, then it would be possible to offer an explanation of the occurrence of the carboxylation of α ketoglutarate.

SUMMARY

1 The rate of carbon dioxide fixation by pyruvate ($Q_{\text{oxaloacetate}}$) was investigated in minced tissues by determining the accumulation of succinate and α -ketoglutarate (a) anaerobically, and (b) aerobically in the presence of malonate.

2 $Q_{\text{oxaloacetate}}$ was computed for the conditions employed to be 1.9 in pigeon liver and, on the average, 1.7 in sheep kidney cortex. In pigeon brain, rat liver, kidney cortex and muscle, guinea pig liver and brain, and sheep liver, heart, brain, spleen, pancreas and lung, no carbon dioxide fixation by pyruvate could be detected by the above methods, and $Q_{\text{oxaloacetate}}$, therefore, must have been less than 1 in these tissues.

3 The rate of carbon dioxide fixation by α -ketoglutarate (Q_{citrate}) was studied by measuring the formation of citrate under anaerobic conditions.

4 In pigeon liver citrate formation was rapid immediately after addition of α -ketoglutarate but ceased within a few minutes. During the first minute Q_{citrate} was 30. Pigeon breast muscle, guinea pig, rat and sheep livers, and sheep heart and kidney cortex all formed citrate from α ketoglutarate. No detectable amounts of citrate were formed by guinea pig, rat or sheep brain, sheep spleen, or rat and sheep testis.

5 Citrate formation was found to be dependent upon the concentration of α ketoglutarate and of bicarbonate and carbon dioxide. It was enhanced by fumarate and inhibited by L. lactate or ammonium chloride. The inhibition by lactate was partly reversed by L. glutamate. Lactate also inhibited citrate formation from oxaloacetate and pyruvate.

6 The significance of the findings is discussed.

I wish to express my gratitude to Prof. H. A. Krebs for his continual aid and advice.

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The Copper-retaining Powers of Different Cacao Soils

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Brun (1945) has shown that soil organic matter (SOM) holds copper in a complex, non-leachable form, the work of Hasler (1943) suggests that most metallic trace elements are so retained in soil, the order of their retention being roughly parallel to their power of forming metallo organic complexes (Mellor & Maley, 1948). From such complexes between trace elements and SOM (Bremner, Mann, Heintze & Lees, 1946) the trace elements presumably become slowly available to plants growing in the soil. In view of the fact that Trinidad cacao soils have been exploitatively cropped for 150 years, a depletion of trace elements in these soils may, in part, be responsible for the general drop in cacao yields apparent over the last 25 years. The present work was therefore begun in order to discover whether 'good' cacao soils (i.e. those on which the decline has been least marked) had a greater power than 'bad' soils to retain a trace element against leaching, and to establish a relation between the percentage of SOM in a soil and the power of the soil to retain a trace element. The trace element chosen was copper, in which cacao is rich (Lindow, Elvehjem & Peterson, 1929).

EXPERIMENTAL

Copper was determined by the method of Sherman & McHargue (1942). Soil organic matter was estimated by the method of Hardy (1929). Because the upper layers of a cacao soil probably play the dominant part in cacao nutrition (McCreary, McDonald, Mulloon & Hardy, 1943), the top 3 in. of soil, air dried and ground to a powder, have been used in all experiments. The soils were divided into 'good' soils and 'bad' soils on the basis of planters' estimates of the cacao yields to be expected from them. Good soils were those estimated to yield 7 or more bags of cacao

per 1000 picquets, bad soils were those estimated to yield less.

The Cu retaining powers of different soils (Fig 1) were determined as follows. A 5 g sample of soil was shaken, over a period of 30 min, with 15 ml 0.5M Na_2SO_4 containing 1.0 mg Cu as CuSO_4 , after which the soil was centrifuged down, and the supernatant liquid poured off. The soil was then again shaken with 15 ml 0.5M Na_2SO_4 containing no Cu and, after 30 min, again centrifuged down, the supernatant liquid being added to the initial one. After 7 such washings, all exchangeable Cu was presumed to have been removed from the soil. The amount so removed was determined by analysis of the bulked supernatant liquids and the amount retained determined by difference.

The data on which Fig 3 is based were obtained in two different ways on each soil.

(a) Either 1, 2, 4, 8, or 16 g of soil were mixed with 50 ml 0.33M Na_2SO_4 containing, as CuSO_4 , either 1.0 mg Cu (for the bad soil) or 5.0 mg Cu (for the good soil). After intermittent shaking over a period of 1 hr the soil was centrifuged down, and the equilibrium concentration of Cu in the supernatant liquid determined.

(b) Either 1, 2, 4, 8, or 10 mg Cu as CuSO_4 , contained in 30 ml 0.33M Na_2SO_4 , were mixed with 5 g soil. Procedure then followed that described in (a).

Preliminary tests showed that equilibration of Cu between soil and solution was attained in less than 5 min. The longer equilibration periods used were adopted as a safety measure.

RESULTS

The relation between the estimated percentage of SOM and the copper retaining power of the soil is shown for thirty-two different cacao soils in Fig 1. Some 2.5% of the estimated SOM of the soils is apparently inactive in retaining copper, moreover, the percentage of the active SOM (total SOM % - 2.5) is not linearly related to copper retention. In fact the curve of Fig 1 closely follows the relation

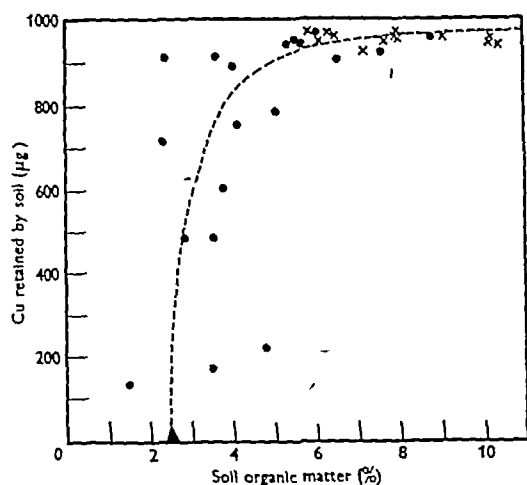


Fig 1 The retention of copper by different cacao soils (5 g samples) treated with 1 mg of copper as CuSO_4 and then well leached with 0.5M Na_2SO_4 . Crosses, good cacao soils, dots, bad cacao soils

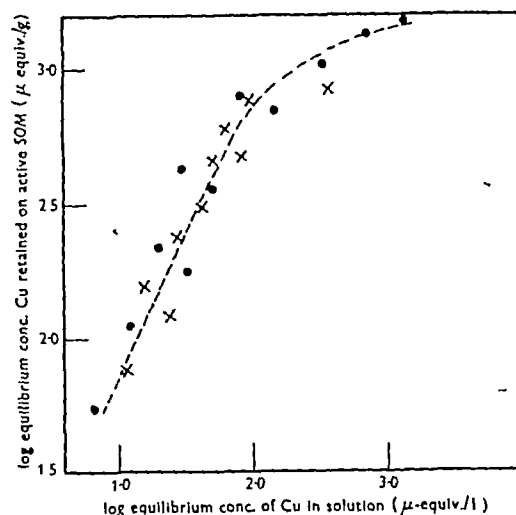


Fig 3 Freundlich isotherm of copper retention, derived from two different cacao soils equilibrated with different concentrations of copper contained in 0.33M- Na_2SO_4 . Dots, data from a bad soil (active SOM, 3.4%), crosses, from a good soil (active SOM, 8.3%)

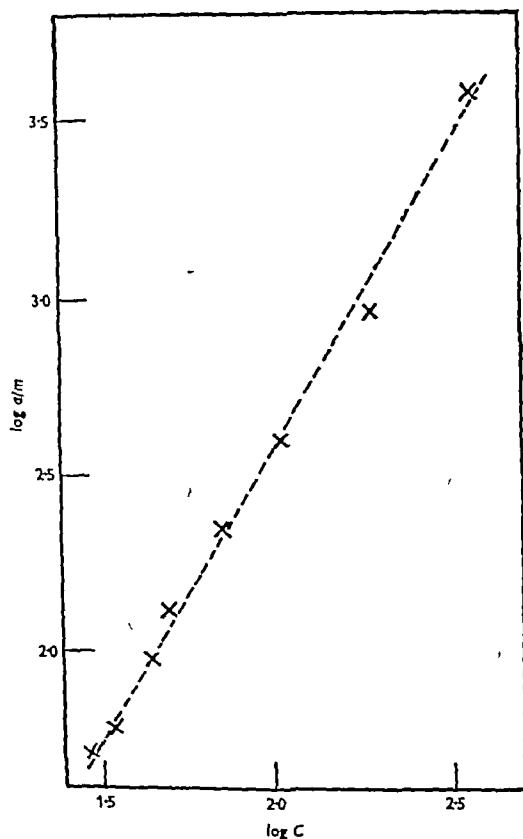


Fig 2 Freundlich isotherm derived from Fig 1, a, copper (μg) retained by soil, m, active SOM (total SOM % - 2.5), C, copper removed by Na_2SO_4 leaching

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Table 1 *Equilibrium distribution of copper between 5 g of a good cacao soil (8.3% active SOM) and various volumes of 0.33M- Na_2SO_4 solution*

(Total copper (as CuSO_4) in soil and solution
5.0 mg (157 μequiv))

Vol of Na_2SO_4 solution (ml)	Equilibrium concn. of Cu in solution ($\mu\text{-equiv/l}$)	Equilibrium concn. of Cu on active SOM ($\mu\text{equiv/g}$)
20	33	377
40	37	376
60	31	374
80	31	374
100	34	371
120	30	370

Cu (mg) retained by soil

Active SOM %

$= \alpha (\text{Cu (mg) released by } \text{Na}_2\text{SO}_4 \text{ extraction})^n$

This is one form of the adsorption isotherm suggested by Freundlich (1930)

$$a/m = \alpha C^{1/n}$$

where a = amount of solute adsorbed, m = weight of adsorbent, C = equilibrium concentration of solute, and α and n are constants. Expressed logarithmically this gives

$$\log a/m = 1/n \log C + \log \alpha$$

If $\log a/m$ is plotted against $\log C$, a straight line should result. A plot of the appropriate data derived from Fig 1 does in fact yield a straight line (Fig 2)

At moderate concentrations of copper in the equilibrium solution (less than 100 $\mu\text{equiv Cu/l}$),

the same equation was found to apply to data from experiments in which the equilibrium distributions of copper between soil and 0.33M-Na₂SO₄ solution were determined for both a good and a bad soil under wide variations of experimental conditions (Fig. 3).

Data in Table 1 show, as might be expected, that the equilibrium concentration of copper in 0.33M-Na₂SO₄ against a given concentration of copper on the SOM is independent of the solution volume.

DISCUSSION

In comparison with good Trinidad cacao soils, bad cacao soils are characterized by a low power to retain copper in complex with the SOM. Although the copper retaining power of a soil is clearly related to its SOM content, the relation is not linear, and nearly all the bad soils lie on or near the steeply descending portion of the curve relating copper retention and percentage of SOM in the soil. Trace-element deficiencies, induced either by leaching or by a too great uptake of trace elements by past cacao crops, are, therefore, far more likely to occur in bad than in good soils.

Although difficulties in estimating small percentages of SOM in all probability caused the scatter

of the points on the descending part of the copper-retention curve (Fig. 1), there is reason to believe that of the total value for percentage of SOM, as given by the method used, some 2.5% represents absorptively inactive SOM. If it be assumed that some 2.5% of the SOM is adsorptively inactive, two widely different soils give the same adsorption isotherm (Fig. 3), a fact that suggests there is no basic difference between the absorptive capacities of the organic fractions of the two soils.

It is reasonable to infer from Fig. 3 that the saturation capacity of SOM is about 1400 μ -equiv Cu/g, to which value the logarithmically plotted Freundlich isotherm, known to be non-linear near saturation of the adsorbent, is asymptotic.

SUMMARY

1 Some bad Trinidad cacao soils have lower contents of organic matter, and lower copper-retaining powers than good soils.

2 The distribution of copper between a soil and a solution in equilibrium with it follows a Freundlich isotherm.

3 The significance of the results is discussed.

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The Secondary Oxidation of Amino-acids by the Catechol Oxidase of Belladonna

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The purpose of this investigation was to examine more fully than has hitherto been done the manner in which a plant polyphenolase is able to oxidize amino-acids in the presence of phenols. It was further hoped to discover which of the common amino-acids are liable to oxidation by the system, and the nature of the oxidation products. *Atropa belladonna* L. was known to possess an active polyphenolase system and was used because the nitrogen metabolism of this plant is under general investigation, and large quantities of reasonably homogeneous material were available.

EXPERIMENTAL

Preparation of the enzyme. Young leafy shoots of belladonna up to about 20 cm. long were harvested in May or June, 120–150 g. were disintegrated in a Waring Blendor with two successive portions of 350 ml. acetone. The acetone was filtered off, and the residue air-dried on a Buchner funnel and ground to a fine powder in a Wiley mill.

This crude powder had considerable polyphenolase activity, 50 mg. powder suspended in 2.5 ml. 0.01M catechol at pH 6 gave an O₂ uptake of over 200 μ l. in 10 min. After three successive extractions with water at room temperature the powder still retained a high oxidase

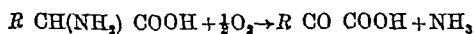
activity, the loss varying somewhat with different batches of powder. The first washing showed oxidase activity equivalent to 1% of the activity of the unwashed powder. The third washing showed little or none.

The first washing gave a strongly positive ninhydrin reaction, but, after three extractions, further washings gave only a faint reaction. The powder therefore provided the desideratum of an active polyphenolase uncontaminated by free amino acids. When dried by a further addition and removal of acetone it gave a stable preparation which kept its activity unimpaired for several weeks at least. In the following work the enzyme was invariably prepared in this way. Samples of the air dry powder were weighed out as required.

Manometry. O_2 uptakes were measured in Warburg manometers using simple flasks with central chambers and single side arms (see Dixon, 1934, fig 11a). CO_2 absorption was provided for, when required, by a roll of filter paper in the central chamber moistened with 20% KOH. In some experiments Keilm cups were used to provide a further possibility of mixing in the enclosed system. Most experiments were run in batches of five with a sixth manometer as thermobarometer. All experiments were of short duration, oxidation of the phenol usually being complete within 1 hr, and the secondary oxidation not being followed beyond a total period of 4 hr. It now seems that the results of earlier investigations of this system may have been vitiated by the dangerously long time interval, up to 40 hr, employed (e.g. Robinson & McCance, 1925).

Ammonia measurements. At the end of the manometric experiments, 0.5 ml was withdrawn from the reaction mixture (total vol. 2.5 ml). This was transferred to the outer circle of a standard Conway unit and the NH_3 content determined in the usual way, using boric acid as the absorbent and mixed bromocresol green and methyl red as indicator (Conway, 1947).

Isolation of the reaction products. It was anticipated that products of amino acid oxidation might be formed according to the general scheme



Attempts were, therefore, made to isolate carbonyl compounds as 2,4-dinitrophenylhydrazones. This was carried out in experiments starting with glycine and alanine. The details of the procedure differ so much in the two examples that they are described separately in the next section.

RESULTS

Oxidation of phenols in the absence of amino acids

Oxidation of catechol. The progress of catechol oxidation by the belladonna enzyme is shown in Fig 1. After 1 hr 105 μl of O_2 had been absorbed by 0.55 mg catechol, corresponding to a mol ratio O_2 /catechol of approximately 1. Further O_2 uptake was slow.

Increasing the concentration of catechol from 0.001 to 0.01M increased the rate of O_2 uptake (Fig 2B). Raising the amount of enzyme present in 2.5 ml from 12.5 to 100 mg also accelerated O_2 uptake (Fig 2A). Increased rates were, however, associated with earlier and more marked fallings

away, so that the optimal conditions for catechol oxidation cannot be simply expressed. The nature of the 'inactivation' is examined further on p 633. In the experiments on amino acid oxidation, catechol was usually required to be present only in catalytic amounts. Concentrations of 0.005 or 0.002M were commonly employed.

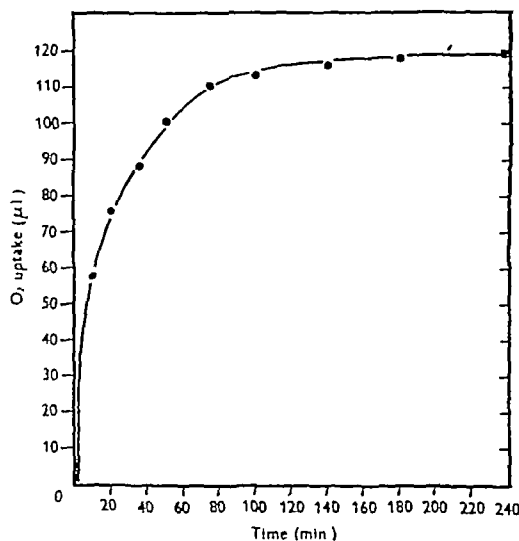


Fig 1 Oxygen uptake over the first 3 hr in the oxidation of 0.002M catechol by belladonna polyphenolase

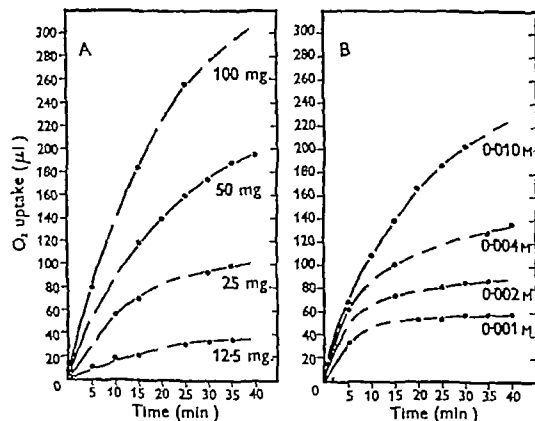


Fig 2 A Oxygen uptake during first 40 min with 0.01M catechol in the presence of varying amounts of enzyme (in 2.5 ml solution) B Oxygen uptake with 50 mg enzyme and varying concentrations of catechol.

The effect of acidity upon the rate of oxidation was examined over a pH range of 4.5–8.3, using phosphate buffers (Table 1). The effect of pH is clearly very slight over the range examined, autoxidation in the absence of enzyme was slow, and consumed only 8 μl O_2 in 60 min even at pH 7.8.

Table 1 *The effect of acidity on the oxidation of catechol by polyphenolase*

(Catechol, 0.005M, 30°)

pH	O ₂ uptake (μl) Time (min)			
	5	15	30	45
4.5	42	97	128	145
5.6	49	105	133	150
6.6	50	100	129	149
7.7	53	106	140	159
8.3	51	107	141	160

Table 2 *O₂ consumption by belladonna polyphenolase with various substrates*

Substrate	O ₂ uptake (μl)								
	(Preparation A 25 mg in 2.5 ml 0.066M phosphate, pH 7.1, 30°)								
Time (min)	10	20	30	40	60	70	90	120	150
0.008M- <i>p</i> Cresol	12	31	47	59	112	—	171	—	—
0.002M Catechol	65	94	100	—	113	—	124	—	—
0.008M Resorcinol	0	0	0	0	0	—	0	—	—
0.008M Hydroquinone	—	—	4	—	16	—	27	—	—
0.008M Phloroglucinol	45	90	127	—	—	—	—	—	—
0.008M Gallic acid	4	8	9	14	18	—	33	—	—
0.002M Adrenaline	19	44	59	74	—	—	—	—	—
(Preparation B 50 mg in 2.5 ml 0.066M-phosphate, pH 7.8, 30°)									
0.002M- <i>N</i> Methyladrenaline	—	—	33	—	40	—	—	—	48
0.004M Aesculin	2	6	8	—	—	10	—	12	—
0.004M Aesculetin	25	39	48	—	—	62	—	69	—
0.002M Catechol	73	86	94	—	—	103	—	115	—

Oxidation of other phenols Several other phenols were substituted for catechol as substrate (Table 2). Typical catechol experiments are included for comparison. The initial rate of oxidation is much faster with catechol than with any of the other phenols examined. Resorcinol is not oxidized and hydroquinone only very slowly, as would be expected. The increasing rate of *p* cresol oxidation with time is also in accord with previous observations by other workers. The unexpectedly vigorous oxidation of phloroglucinol was confirmed in several further experiments, the O₂ consumption amounted to about 3 atoms/mol phloroglucinol.

The oxidation of adrenaline (Table 3) was examined at different acidities and, unlike that of catechol, was found to be markedly sensitive to

Table 3 *The effect of acidity on the oxidation of adrenaline by polyphenolase*

(Adrenaline, 0.002M, 30°)

pH	O ₂ uptake (μl) Time (min)					
	10	20	30	40	120	150
4.1	0	8	10	18	41	68
6.0	11	26	36	47	85	112
7.1	19	44	59	74	142	181
7.8	36	59	93	112	211	249

changes of pH. The final O₂ uptake greatly exceeds 2 atoms O₂/mol adrenaline and melanin-like pigments are formed (cf Blaschko & Schlossmann, 1940).

Inhibitors. Since the enzyme is likely to have copper as its prosthetic group, the action of heavy-metal inhibitors was investigated over a range of concentrations. Cyanide (Table 4) was used at 0.01–0.001M and sodium diethyldithiocarbamate ('dieca') (Table 5) at 0.005–0.0002M. CO (Table 6) was tried at atmospheric pressure in a mixture of CO (approximately 80%) and O₂. Since no CO₂

production was to be expected, KOH was not put into the inner cups. There was not, therefore, any risk of loss of cyanide from the reaction mixture by distillation into alkali. The enzyme is markedly

Table 4 *Inhibition of catechol oxidation by cyanide at pH 6*

(25 mg polyphenolase, phosphate buffer, 2.5 ml 0.005M catechol, 30°)

Cyanide concn (M)	O ₂ uptake (μl) Time (min)				
	5	10	20	30	45
0.0	51	76	98	115	133
0.001	30	53	81	102	125
0.0013	17	31	48	67	89
0.002	10	14	21	27	40
0.01	0	0	0	0	0
% inhibition at 0.001M	41	30	17	11	6

resistant to 0.001M cyanide. Plant polyphenolases have frequently been found to have a high resistance (Deb & Roberts, 1940; Roberts, 1941) and there seems to be much variation with species (Wiand & Sutter, 1930).

With diethyldithiocarbamate, inhibition is more pronounced than with cyanide, and is stronger in the weaker preparation in spite of the larger ratio of solid matter to dithiocarbamate. This is to be

Table 5 *Inhibition of catechol oxidation by diethyldithiocarbamate*

(Preparation A, 25 mg, preparation B, 50 mg, each in 2.5 ml. 0.060M phosphate, pH 6, 2.5 ml. 0.005M catechol, 30 °)

Diethyldithiocarbamate concn (M)	O ₂ uptake (μl.) Time (min)									
	Preparation A					Preparation B				
	5	10	15	25	40	5	10	15	25	40
0.0	47	68	84	107	121	37	58	69	85	96
0.0002	41	60	73	96	115	25	41	51	69	84
0.001	25	38	51	68	85	13	23	30	41	51
0.002	—	—	—	—	—	11	23	30	39	48
0.005	15	26	32	45	51	2	12	17	23	25
% inhibition at 0.001M	50	44	39	36	30	65	60	56	52	47

Table 6 *Inhibition of the polyphenolase activity of freshly expressed juice of belladonna by carbon monoxide*

(2 ml. juice, catechol 0.005M, total vol 2.5 ml)

Conditions*	O ₂ uptake (μl.) Time (min)					
	5	10	15	20	25	30
Air	100	160	208	229	239	243
80% CO, 20% O ₂ (a)	54	100	149	177	198	208
(b)	60	115	151	179	206	215
(c)	47	100	137	173	—	—

* Catechol tipped from side arm (a), 20 min, (b), 40 min, and (c), 60 min after treatment with gas mixture

Table 7 *Oxidation of ascorbic acid by polyphenolase*

(25 mg polyphenolase in 2.5 ml. 0.002M catechol, phosphate buffer pH 6)

Substrate	O ₂ uptake (μl.) Time (min)							
	5	10	20	30	45	60	90	125
0.002M Catechol	47	60	86	88	98	103	107	115
0.002M Catechol + 3.52 mg ascorbic acid	48	86	123	139	169	191	214	237
3.52 mg ascorbic acid	2	7	15	15	20	38	56	76

expected if the activity of the system depends upon the amount of copper present, since diethyldithiocarbamate is an almost specific copper precipitant.

In the experiments with CO, it was shown that the inhibitions could not be reversed by exposure to bright light. These results are in agreement with those of Kubowitz (1937) for potato polyphenolase.

Oxidation of ascorbic acid. The system is capable of acting as a continuous oxidation mechanism, and this was observed by providing ascorbic acid as substrate (Table 7). Glass distilled water was used for all solutions. The slow continuous uptake of O₂ with ascorbic acid alone may indicate the presence of a direct ascorbic acid oxidase in the belladonna powder. The O₂ uptake with ascorbic acid and catechol present together exceeds the sum of the O₂ uptakes when they are present separately. It can, therefore, be concluded that the system catalyzes oxidation of ascorbic acid with catechol acting as redox body.

The oxidation of amino acids and peptides in the presence of catechol

Oxygen uptake with catechol + glycine. Numerous experiments have been performed with mixtures of catechol and glycine. A typical set of results is in Table 8. The principal features evident in these results have been found in all experiments of the same kind. Glycine by itself is not oxidized, even slowly. Added to catechol it does not increase the rate of O₂ uptake very much during the first 20 or 30 min, by which time catechol oxidation is usually well advanced. During the second hour, the mixture of catechol and glycine continues to absorb O₂, and this still goes on in experiments of longer duration. It has been followed up to 4 hr from the start (Fig. 3). The rate of amino acid oxidation is always slow, relative to the initial rate of catechol oxidation, even under the most favourable conditions. Uptakes of 80–100 μl O₂ are recorded in the first 10 min with

Table 8 O_2 uptake by polyphenolase with catechol and glycine

(25 mg polyphenolase in 25 ml 0.066M phosphate pH 6, catechol, 0.002M, glycine, 0.02M, 30°)

Substrate	O_2 uptake (μ l.) Time (min)								
	5	10	20	30	45	60	75	100	120
Glycine	0	0	0	0	0	0	0	0	0
Catechol	65	92	108	117	120	122	124	124	124
Glycine + catechol	56	84	104	116	125	135	142	148	157

catechol alone, but a period of about 3 hr is necessary to raise O_2 consumption in the presence of glycine by the same amount, once catechol oxidation is complete. The time lag before an extra O_2 uptake

240 min showed that NH_3 formation at pH 7.8 was progressive (Fig. 4). After 150 min 198 μ l. NH_3 had been produced. This is approximately twice the

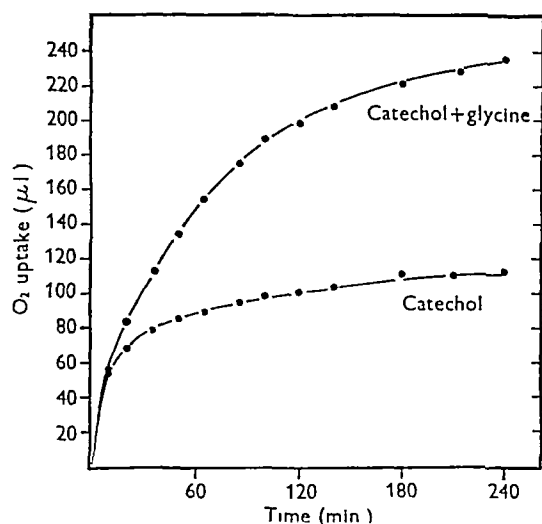


Fig. 3 Oxygen consumption over 4 hr with 0.002M catechol (lower curve) and 0.002M catechol + 0.02M glycine (upper curve)

becomes evident is often more pronounced than in Fig. 3, and strongly suggests that the amino acid is not being oxidized by a simple oxidation product of catechol, such as *o*-benzoquinone.

Ammonia formation with catechol + glycine A series in which different flasks were opened and sampled after successive time intervals from 20 to

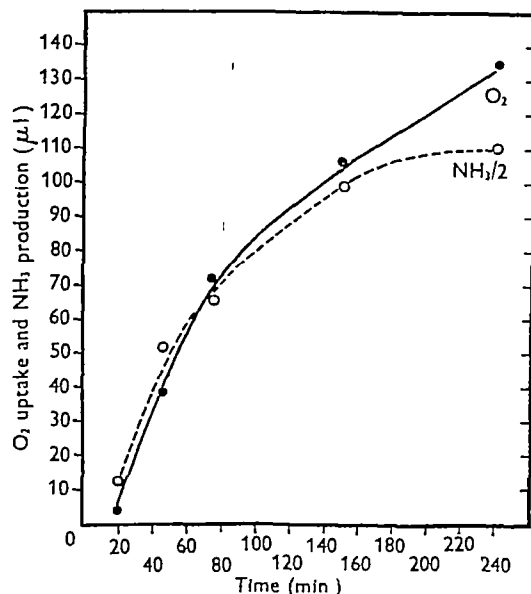


Fig. 4 Oxygen consumption and ammonia production with 0.002M catechol + 0.02M glycine. The ordinates represent μ l. O_2 consumed and μ l. ammonia/2 produced.

difference between the volume of O_2 consumed and that required for complete oxidation of the catechol present ($227 - 130 = 97 \mu$ l. O_2). In other words, 1 mol. NH_3 had been released for each atom of extra O_2 consumed, as predicted by the equation on p. 627.

Table 9 O_2 uptake and NH_3 production after incubation of polyphenolase with catechol and glycine at various pH values

(25 mg polyphenolase in 0.066M phosphate, catechol, 0.002M, glycine, 0.02M, 30°)

pH	Time (min)	O_2 uptake (μ l.)			Ammonia production/2 (μ l.)
		Catechol only	Catechol + glycine	Extra uptake due to glycine	
4.2	210	127	134	7	6
6.0	120	124	157	33	33
7.1	150	133	250	117	100
7.1	180	115	255	140	116
7.8	225	120	328	206	130
7.8	240	130	264	134	110

After 240 min, the ratio NH_3/O_2 had fallen off, presumably owing to the entry of the NH_3 into side reactions. Other results of experiments lasting 2-4 hr are shown in Table 9.

Absence of carbon dioxide production Omission of KOH from the central cups produced no effect upon the pressure changes recorded at pH 7.1 with 0.002M catechol and 0.02M glycine. No CO_2 is released, therefore, but some might be retained in the buffer. When 0.5M-HCl was tipped into the reaction mixture after an uptake of 258 μl O_2 and NH_3 production of 220 μl in 3 hr, there was an output of 81 μl CO_2 , an output of 77 μl CO_2 was obtained when acid was similarly tipped into a suspension of enzyme in buffer without added substrates. No CO_2 is produced, therefore, in the oxidation of catechol + glycine by our polyphenolase preparation.

The effect of acidity on the oxidation of glycine Although the enzymic oxidation of catechol was found to be almost unaffected by changes of pH between 4.5 and 8.3, the secondary oxidation of glycine was markedly sensitive to changes in pH. This is in agreement with Oparin's (1927) observation that the rate of oxidation of amino acids by chlorogenic acid increases between pH 7 and 12.

Formation of glyoxylic acid Enzyme powder (250 mg) was incubated with 5 ml. 0.01M catechol, 12.5 ml. 0.1M glycine and 12.5 ml. phosphate buffer (pH 7.8), and kept at 30° with occasional shaking. A red coloration developed rapidly, changing to dark purplish brown at the end of 3.5 hr. The solid enzyme was then filtered off, the purplish filtrate gave no reactions for proteins. On dilution of a small portion to a pale colour and addition of free tryptophan, a strong blue colour (Hopkins Cole reaction) was given.

The remainder of the solution was treated with 0.5 vol of 0.1% 2,4-dinitrophenylhydrazine in 2N HCl, and allowed to stand in the refrigerator overnight. A dark granular solid which settled out was removed by decantation, and washed twice with 2N HCl, twice with water and allowed to dry. After a short extraction with ethyl acetate, the bulk of the solid went rapidly into a yellow solution leaving a heavily coloured residue. The solution was decanted, and the ethyl acetate allowed to evaporate at room temperature. A yellow-orange crystalline product remained which gave a red colour with ethanolic NaOH and a very strong red colour with aqueous KOH. The crystals were long, feathery, branching plates, m.p. 200°. Mixed melting point with the reagent (2,4-dinitrophenylhydrazine) was depressed to 179°, mixed melting point with hydrazone freshly prepared from glyoxylic acid, 201°. Glyoxylic acid had, therefore, been isolated in identifiable quantities from the digest.

Oxidation of catechol and alanine The oxidation of alanine was examined in the same way as that of glycine. At pH 6, O_2 uptake was not measurable over 4 hr. It was demonstrable at pH 7.1 and 7.8, but much slower than with glycine (Table 9). NH_3 production was correspondingly small, and the calculation of NH_3/O_2 ratios liable to too great an error to be of any value.

Formation of pyruvic acid Only very small yields of the probable oxidation product, pyruvic acid, could be expected.

In the light of previous experience (James & James, 1940) and after some preliminary trials, small yields were obtained by the following method. Enzyme extract (10 ml) was incubated at 30° with 0.005M catechol, 9 mg alanine, an excess of CaCO_3 and toluene were added and the vol. made up to 20 ml. After 16 hr the digest was distilled under reduced pressure. The distillate was discarded and the digest acidified with dilute H_2SO_4 , filtered and again distilled *in vacuo*. About 20 ml. distillate was collected, and 10 ml. 0.2% 2,4-dinitrophenylhydrazine added (less would have been better). After standing, the yellow solution was extracted with ethyl acetate. The ethyl acetate solution was extracted twice with half saturated Na_2HPO_4 solution. The aqueous layer was separated and acidified with 2N HCl and then extracted with ethyl acetate. A further transfer was made into 0.066M Na_2HPO_4 which took only a small fraction of the coloured material from the ethyl acetate. The dilute alkali fraction was separated, acidified and returned to ethyl acetate. This was allowed to evaporate. A few yellow crystals were obtained. After further solution in dilute sodium phosphate, and recrystallization from ethyl acetate, crystals were obtained as diamond shaped bright yellow platelets with m.p. 215°. Melting point of freshly prepared hydrazone of redistilled pyruvic acid, 216°.

Oxidation of other amino acids and dipeptides The other amino acids are oxidized much more slowly than glycine, and the alanyl dipeptide more slowly than the glycol dipeptides (Table 10). For each atom of O_2 consumed in the oxidation of glycine there is rather less than 1 mol of NH_3 released, probably due to the utilization of NH_3 in some side reaction. With the much slower rates of oxidation of the other amino acids the discrepancy becomes much larger.

Absence of an amino acid dehydrogenase from the insoluble enzyme preparation

The observation that the secondary oxidation is highly sensitive to changes of pH might suggest that it was catalyzed by an amino-acid dehydrogenase. The possibility can, however, be discounted for the following reasons: (i) The conditions of preparation of the enzyme are such that no dehydrogenase could be expected to survive. (ii) Malachite green, a reputed dehydrogenase poison (Boswell & Whiting, 1938), was found to inhibit the oxidation of catechol alone and catechol + glycine only slightly at a concentration of 0.002M. (iii) The polyphenolase is unusually resistant to heat for an enzyme, and heating at 100° for 5 min diminished the oxidation of catechol + glycine as well as of catechol alone only to a very slight extent. (iv) The oxidation can be reproduced, including NH_3 production, if O_2 and polyphenolase are replaced by $\text{K}_2\text{Fe}(\text{CN})_6$, and no enzyme is present in the system at all. (v) Secondary amines are not oxidized, but do form a coloured complex (Beever & James, 1948), and there is no diminution in the rate of amino-acid oxidation if the enzyme is filtered off after forming the coloured complex with a secondary amine. On addition of amino-acid, a normal rate of oxidation is catalyzed by the coloured complex. Since the enzyme is exhaustively extracted beforehand with water, none can be supposed to remain behind in solution. From these facts it seems clear that no enzyme was catalyzing the secondary oxidation of the amino acid in our extracted system, but this does not necessarily rule out the existence and participation of such an enzyme in the intact cell.

Table 10 O_2 uptake and NH_3 production after incubation of polyphenolase with catechol and various amino-acids

(25 mg polyphenolase, 0.002M catechol + 0.02M-amino acid, phosphate buffer pH 7.8, vol. 2.5 ml, 30°)

Amino acid	O_2 uptake (μ l)			Ammonia production/2 (μ l.)
	Catechol only	Catechol + amino-acid	Difference	
Glycine	130	264	134	110
DL-Alanine	120	150	30	9
DL-Phenylalanine	130	169	39	21
DL-Aminobutyric acid	130	154	24	12
L(?) Methionine	130	126	—	16
L-Valine	130	103	—	7
L-Leucine	130	191	61	37
DL-Isoleucine	130	174	44	13
L(?) Aspartic acid	130	153	23	7
L-Glutamic acid (163 min)	133	179	46	18
L-Histidine	130	152	22	9
L(?) Tryptophan	130	163	33	13
Glycylglycine	130	211	81	45
Glycyl L(?) leucine	130	192	62	43
DL(?) -Alanylglycine	130	133	3	8

Formation and activity of a coloured complex

When catechol alone is oxidized by the enzyme a pale yellow colour is produced which slowly turns brown. With the low concentrations of catechol employed in these experiments the colour was rarely deeper than pale straw, even after 4 hr. Addition of amino acid caused the rapid formation of a characteristic intense red irrespective of the rate at which the particular amino acid was oxidized. Alanine produced a full development of the colour at pH 6, although its O_2 uptake was negligible at this pH and much less than that of glycine at pH 7.8 (Table 10).

A similar reaction is given with amino acids present in belladonna extracts from resting tissues. On addition of catechol to a suspension of the unwashed acetone preparation of the enzyme, the typical red colour develops. The enzyme, which has been washed with water until the washings are no longer capable of giving a ninhydrin reaction, fails to form the colour when incubated. For this reason the washed insoluble enzyme was particularly convenient material for the present investigation. It was observed that colour formation is much more rapid at pH 7.8 than at pH 6. This effect could also be reproduced if the enzyme and O_2 were replaced by inorganic oxidizers. Treatment with ceric sulphate in acetate buffer at pH 5 oxidizes catechol immediately (Ball & Chen, 1933), but we found that colour formation with glycine or proline was slow, extending over 15 min. Oxidation at pH 7.8 with potassium ferricyanide in phosphate buffer led to colour formation almost immediately. This observation is significant in the light of the conclusion of Nelson & Dawson (1944) that conversion of *o* benzo-

quinone to *p* hydroxy-*o* quinone is relatively slow below pH 7.

Accurate correlation of the amount of colour produced with the amount of oxidation performed is not very easy if enzyme and O_2 are the agents. It is more readily carried out using potassium ferricyanide as oxidizer. Two mol of ferricyanide are equivalent to 1 atom of O_2 and oxidize 1 mol catechol to *o* quinone. It has been shown on p. 627 that the complete oxidation of the catechol molecule requires 2 atoms O_2 ($\equiv 4K_3Fe(CN)_6$). By means of measurement in a Spekker absorptiometer it was found that $4K_3Fe(CN)_6$ is also needed for the development of the full colour from 1 mol catechol in the presence of an excess of glycine.

Colour formation is reversible. The colour is bleached by ascorbic acid or by magnesium + dilute acetic acid and restored by shaking in air. It is also bleached by reduction with bisulphite. Separation of coloured and leuco forms of the compound has been obtained by shaking a deeply coloured solution with charcoal for a few minutes at room temperature, and then filtering. The filtrate is completely colourless, but on standing in air becomes coloured again. The amount of colour formed by the second oxidation is small compared with the original. In this way, the coloured compound may be produced slowly from the leuco form, even in the absence of enzyme, at a stage when catechol is no longer present, and it may be bleached with hydrogen donors. The leuco and coloured compounds are likely, therefore, to resemble phenolic and quinonoid forms respectively, and may be capable of transferring hydrogen reversibly.

The coloured complex, as produced by the action of potassium ferricyanide on catechol and glycine,

is able to effect oxidation of glycine in the absence of any enzyme. With ferricyanide, catechol and glycine present in the molecular proportions of 4 : 1 : 10 (0.55 mg catechol in 2.5 ml phosphate buffer pH 7.8) an uptake of O_2 and production of NH_3 were clearly demonstrable (Table 11). Both these were

Competition between catechol and reduced coloured complex. After a period of about 60 min, oxidation of catechol becomes very slow. This is not due to inactivation of the enzyme, since on addition of fresh catechol the rate of O_2 uptake again becomes rapid (Fig 5, curve I). If glycine is present with

Table 11 O_2 uptake and NH_3 production under varying conditions of oxidation

(Wts of reagents, a, b, c, d in mg, phosphate buffer pH 7.8, total vol of reaction mixture, 2.5 ml)

Exp	Wt of reagents (mg)				Molecular ratio b c d	O ₂ uptake (μl.) Time (min)										Ammonia production (μl.) 240
	Enzyme (a)	K ₃ Fe(CN) ₆ (b)	Catechol (c)	Glycine (d)		10	20	30	45	60	75	155	180	240		
1	—	6.6	0.55	3.75	4 1 10	—	10	36	61	88	110	197	220	262	54	
2	25	6.6	0.55	3.75	4 1 10	11	24	50	81	111	140	264	288	314	87	
3	25	6.6	1.10	—	4 2 0	48	63	74	78	84	89	112	118	137	10	
4	25	—	0.55	—	0 1 0	48	63	73	78	90	95	113	113	123	8	
Control, reagents only at zero time															9	

higher in the presence of enzyme, indicating that the secondary oxidation is indirectly accelerated by the enzyme, though autooxidation without it is quite marked. The experiment shows unequivocally that the coloured complex can bring about the breakdown of glycine directly without the action of any enzyme

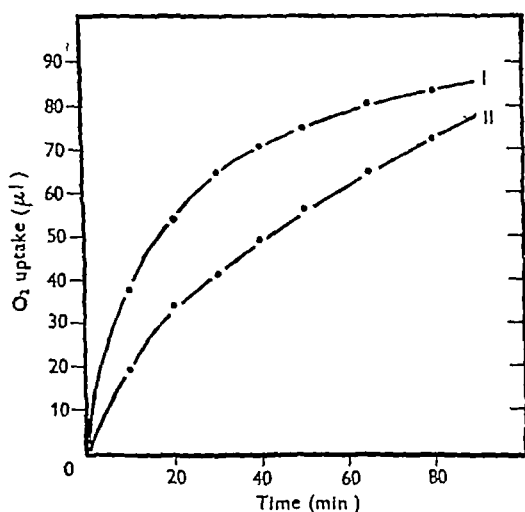


Fig 5 Oxygen consumption after an initial period of 60 min which is not included in the graph. Curve I: 0.002M catechol present at start and a second equal amount added at 60 min; curve II: 0.002M catechol + 0.002M glycine present at start, an equal amount of catechol added after 60 min.

(Table 11, line 1) The increased breakdown of glycine in the presence of the polyphenolase (line 2) is due to faster formation and regeneration of the coloured complex, and it has already been shown (p. 631) that no second enzyme intervening between the coloured complex and the amino acid is likely to be present in our preparations.

catechol from the beginning of the experiment, a moderate rate of O_2 uptake continues steadily over a long period. The addition of extra catechol to the mixture after 60 min causes an increase in the rate (Fig 5, curve II), which is not as great as the increase obtained in the absence of the amino acid. It cannot, therefore, be supposed that the presence of the amino acid tends to retain the catechol in the oxidizable form, as would occur if it were itself being oxidized by the initial catechol oxidation product (o-quinone). A more probable explanation of these results is that the reduced form of the coloured complex competes with catechol for the enzyme, and is itself oxidized, but more slowly than catechol.

Relation of amino acid oxidation to amino-acid concentration. The extent of amino acid oxidation can be estimated either by the NH_3 produced, or by the O_2 consumed over and above that required for oxidation of the catechol. Data are available from a series of experiments in which NH_3 and O_2 changes were recorded with varying amounts of amino acid. In all of them 25 mg of the same enzyme preparation were used with 0.002M catechol in 2.5 ml phosphate buffer at pH 7.8. The experiments were carried on in the manometers for 4 hr. The results are expressed in Figs 6 and 7 by plotting μ l NH_3 and O_2 against the mol ratio glycine/catechol. Both for NH_3 and O_2 the relation is linear from 1 to 10 mol glycine/mol catechol. In neither graph does the line spring from the origin. Some NH_3 is produced from the enzyme in the absence of added glycine, and no increase is recorded with additions of 0.5 and 1.0 mol/mol catechol. Values < 1 mol are clearly off the line and their departure is statistically significant. The mean deviation of three experiments at zero concentration of glycine from the regression line of NH_3 production in glycine concentration is 22.4, with a calculated probability of being on the line of only 0.001. The results of this experiment indicate that there is no

oxidation of amino acid until there is an excess of glycine over 1 mol for each mol of catechol, or, in other words, that 1 mol of amino acid enters into combination with the catechol oxidation products before oxidation of amino acids can occur

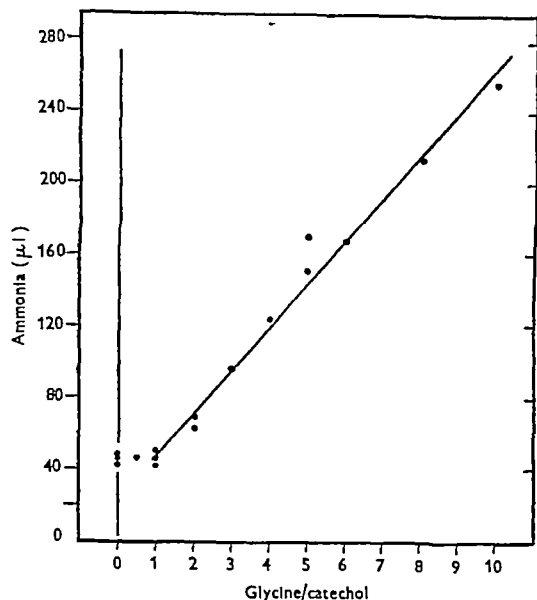


Fig 6 Ammonia production with varying molar concentrations of glycine Catechol concentration 0.002M throughout

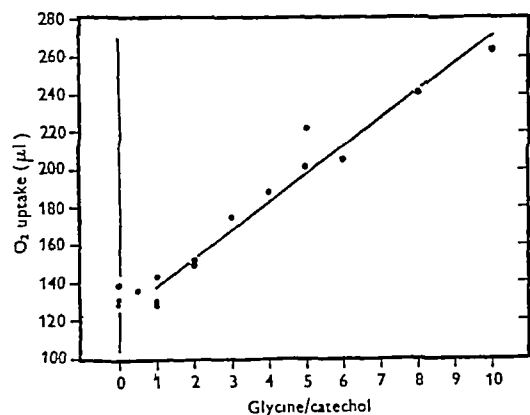


Fig 7 Oxygen consumption with varying mol. ratios of glycine and catechol, the catechol concentration was 0.002M

The data for O₂ consumption are consistent with this idea, though they are not in themselves so conclusive, on account of the greater error involved in allowing for the O₂ consumed in the primary catechol oxidation. Reference to Fig 7 shows that the results for 0 and 0.5 mol glycine/mol catechol again appear to be off the regression line. There are three results available for zero concentration with a mean deviation of 10.3 from the value predicted by the regression. Calculation by the usual methods shows

a probability of its belonging to the curve of only 0.07 (about 1 in 14.5). There is no evidence of additional O₂ consumption until 1 mol of glycine has been fixed for every molecule of catechol originally present.

Substitution of adrenaline for catechol leads to an interesting result. The effect on the O₂ uptake of adding glycine to the adrenaline is masked by the complexities of the further oxidations of adrenaline itself (p. 628). NH₃ is not released in these adrenaline reactions, but is formed when glycine is added. The effect of varying the concentration of glycine is shown in Fig 8, and it is clear that the curve proceeds linearly to the zero value. In other words, the

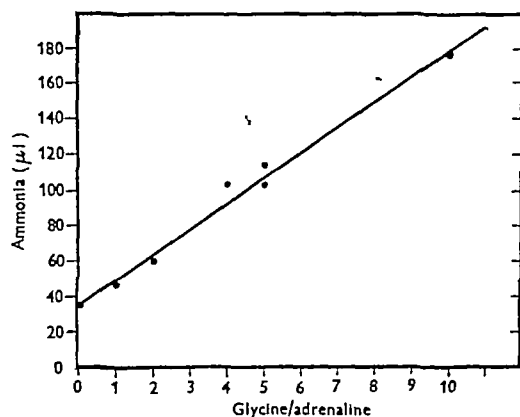


Fig 8 Ammonia production from glycine by polyphenolases and adrenaline. Adrenaline concentration 0.002M, mol. ratios of glycine/adrenaline varying from 0 to 10

amino acid is oxidized without the prior formation of a coloured complex involving the amino acid. The place of the coloured complex is taken by the adrenochrome produced by the oxidation of adrenaline (Blaschko & Schlossmann, 1940), and the combination of 1 mol of amino acid with the intermediate oxidation product is no longer necessary before oxidation of glycine can take place.

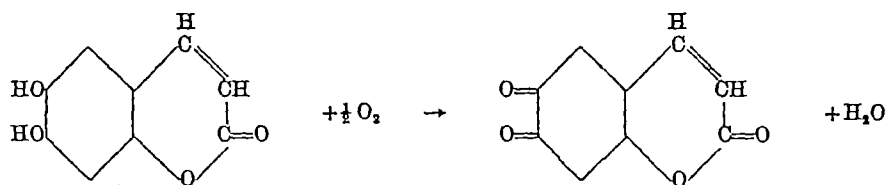
N-Methyladrenaline and aesculetin are also oxidized by the polyphenolase at readily measurable rates (Table 2), but they do not form a coloured compound like adrenochrome either alone or with addition of amino acid. They are also unable to set up an oxidation system for glycine.

DISCUSSION

The secondary oxidation of amino acids by polyphenolase systems has been assigned considerable importance in the nitrogen metabolism of plants, but has not been examined extensively. The general characteristics of the system, as it exists in *Atropa belladonna*, are shown by the experiments described above. The enzyme oxidizing the polyphenol can be prepared in a highly active, insoluble form by

precipitation with acetone. From this the remaining polyphenols and amino acids can be washed out with water, leaving a large proportion of the original polyphenolase activity in the precipitate. Pure catechol is vigorously oxidized by this precipitate with the uptake of approximately 2 atoms O_2 /mol catechol oxidized, and the greater part of the enzymic activity remains unimpaired when this has been done.

In the absence of other oxidizable substances, brown condensation products of the catechol appear. The oxidation of catechol is little affected by changes of pH over the range 4.5–8.3, but other polyphenols



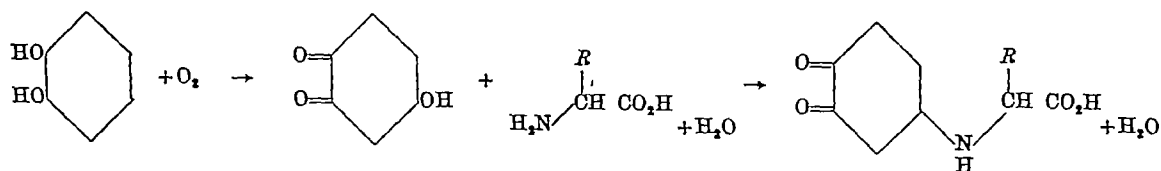
may behave differently, since the oxidation of adrenaline was found to be much faster at pH 7.8 than at 4.1, and the total uptake of O_2 much greater than with catechol. The enzyme is poisoned by 0.001M diethyldithiocarbamate and, therefore, probably contains copper, but, like many other plant polyphenolases, it is markedly resistant to 0.001M cyanide. Catechol is reduced again by ascorbic acid, for example, and a continuous oxidation system with catechol as carrier can be set up. It would be natural to suppose at first that the progressive oxidation of amino acids was similarly achieved, but the results obtained suggest a rather more complex mechanism. On addition of amino acid to the enzyme catechol mixture a rich red coloration develops, altogether different from the brown colours with catechol alone. Similar colours are given by some other primary amines. The addition of one equivalent of amino acid does not result in the release of any ammonia, and with further additions of amino acid there is a linear increase in the amount of ammonia released

is also faster at pH 7.8 than in slightly acid solutions, whether inorganic oxidizers or enzyme + O_2 were used. It appears probable, therefore, that the coloured compound is formed by the reaction of a molecule of *p*-hydroxy-*o*-quinone with a molecule of amino acid. This amino acid molecule is not subsequently released in oxidized form. Its condensation with the quinone precedes the oxidation of further amino acid.

Condensation of the amino group at the 4 position of the nucleus is confirmed by the inability of aesculetin, in which this position is already occupied,

to form a coloured complex, even when oxidized (p. 634). The O_2 uptake in this case was equivalent to less than 1 atom of O_2 /mol of aesculetin. It would, therefore, appear that no hydroxyl group is attached and no condensation occurs in positions adjacent to the *o*-hydroxyl groups. Further confirmation is afforded by the behaviour of adrenaline. On oxidation it forms adrenochrome (Green & Richter, 1937; Blaschko & Schlossmann, 1940), which is not unlike our coloured compound in tint and intensity of colour. Ring closure occurs at the 4 position, no ammonia is released, but the adrenochrome formed is able to oxidize glycine with liberation of ammonia from the amino acid. With the fully *N*-substituted *N*-methyladrenaline which is a tertiary base, no such cyclization can occur and the oxidation of glycine is not catalyzed.

Assuming, for the above reasons, that the coloured compound is formed by condensation of amino N at position 4 on the nucleus, we may write its formation as follows:



The oxidation of 1 mol of catechol to *o*-benzoquinone requires only a single atom of O_2 or its equivalent. Reasons have been advanced by Nelson & Dawson (1944), using potato tyrosinase, for supposing that the consumption of the second O_2 atom leads to the formation of *p*-hydroxy-*o*-quinone, which occurs slowly at pH 4 and faster at pH 7. It is significant that colour formation in our experiments

The coloured condensation product, which seems at present to be best formulated as a substituted *p*-amino-*o*-quinone, does not condense rapidly like *p*-hydroxy-*o*-quinone, but becomes the actual oxidizer of the amino acid. The participation of the enzyme may be limited to its initial formation. Once the coloured compound is formed, its reduction by the amino acid is non-enzymic, and its reoxidation in

the presence of oxygen may occur spontaneously also. Nevertheless, the reduced coloured compound is capable of uniting with the enzyme surface (p 633), where its reoxidation appears to be accelerated.

SUMMARY

1 From *Atropa belladonna* leaves a catechol oxidase has been isolated which is highly active in an insoluble form. It was obtained free from polyphenols and amino acids.

2 It was found to oxidize catechol with the rapid uptake of approximately 2 atoms of oxygen/mol catechol, further oxygen uptake was slow.

3 Phloroglucinol, *p* cresol, adrenaline, *N* methyl adrenaline and aesculetin were also oxidized rapidly, hydroquinone and gallic acid more slowly, aesculin very slowly and resorcinol not at all.

4 Variation of pH between 4.5 and 8.3 had little effect on the rate of oxidation, cyanide inhibited very slightly, but diethyldithiocarbamate more strongly. Carbon monoxide inhibited, and the inhibition was not reversible by light.

5 Secondary oxidation of glycine took place with release of ammonia and uptake of additional

oxygen. The volume of ammonia produced was approximately double the volume of extra oxygen consumed. Glyoxylic acid was isolated from the reaction products. No carbon dioxide was released. Pyruvic acid was similarly obtained from alanine, but the reaction was much slower than that with glycine.

6 Other amino acids and dipeptides were oxidized much more slowly than glycine, and some not at all.

7 Formation of a red colour preceded the secondary oxidation. Once it is formed, its reduction by the amino acid is non enzymic, and its reoxidation in the presence of oxygen may occur spontaneously. Nevertheless, the reduced coloured complex is capable of uniting with the enzyme surface, and its reoxidation may be accelerated. Reasons are given for supposing that the coloured complex, probably a *p* amino *o* quinone, is the immediate oxidizer of the amino acid, and that the role of the polyphenolase is limited to its formation and possibly also its regeneration in the system.

The authors had the pleasure and profit of frequent discussion with Dr E. M. Trautner while this work was in progress. They are indebted to Dr H. Blaschko for a sample of *N* methyladrenaline.

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The Behaviour of Secondary and Tertiary Amines in the Presence of Catechol and Belladonna Catechol Oxidase

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The results presented in the preceding paper (James, Roberts, Beevers & de Kock, 1948) showed that a polyphenol oxidase from belladonna, using catechol as an intermediate, was capable of oxidizing certain primary amino acids. The present paper describes the effects of the same enzyme system on secondary and tertiary amines, and, in addition, the oxidation of glycine which occurs when it is added to a complex of catechol and secondary amine in the presence of the enzyme.

EXPERIMENTAL

The enzyme was prepared as described in the preceding paper (James *et al.* 1948) and the manometric technique was essentially the same, the experiments were carried out at 30°, and the solutions were buffered to pH 7.8 by the addition of 0.1M phosphate mixtures. When two reactants were to be added separately to the main mixture in the manometer vessels, Keilin cups were employed.

RESULTS

The reactions of secondary amines with the enzyme catechol system

No O_2 uptake was observed when secondary amines, e.g. dimethylamine, were added alone to an enzyme suspension and no colours developed. When catechol was also added a strong colour quickly appeared. It was noticed that, with secondary amines, this colour was different from that developed with primary amines and amino acids. Thus, whereas methylamine, glycine, alanine, leucine, aspartic acid, glutamic acid and other primary amino acids produced reddish brown colours, the secondary amines, dimethylamine, proline, sarcosine and nortropine gave rise to deep royal-purple colours. Although the colours produced were different, it was clear that the secondary amines were reacting with an oxidation product of catechol in a way analogous to that which occurs with amino acids, and manometric experiments were carried out to discover if any oxidation took place under these conditions.

Solutions of the desired amine (0.5 ml, 0.1 M) were placed in the main chamber of manometer vessels with 50 mg enzyme suspended in 1.5 ml. 0.086 M phosphate buffer at pH 7.8, and 0.01 M catechol (0.5 ml) was placed in the side arms. Control runs with water instead of secondary amine were set up. After 15 min shaking at 30°, the catechol was tipped in and the O_2 uptakes measured over 200–240 min.

Fig 1 A and B shows the results obtained with the secondary amines listed above. In the controls, the catechol was oxidized vigorously in the usual manner, and the O_2 uptakes, which were very steep at first, flattened out as the substrate was used up. On addition of the secondary amine there was no significant increase in the rate of O_2 uptake, even after a period of 4 hr. Although royal purple colours were developed in the presence of the secondary amines, it was obvious that the formation of the coloured complex did not involve any extra O_2 uptake, and, furthermore, there appeared to be no oxidation of the secondary amines under conditions in which amino acids are oxidized.

The reactions of tertiary amines with the enzyme-catechol system

As differences in behaviour of amino acids and secondary amines had been encountered, some experiments with tertiary amines were carried out. In particular, it was of interest to know whether the belladonna enzyme would oxidize the belladonna alkaloid, *laevo* hyoscyamine, and the basic alcohol, tropine, of which it is the tropyl ester. Similar experiments to those described above for secondary amines showed that no colours were developed when catechol was oxidized by the enzyme in the presence of *laevo* hyoscyamine, tropine or

trimethylamine neutralized with hydrochloric acid. Manometric determinations established that the tertiary amines were not themselves oxidized (Fig 1 C).

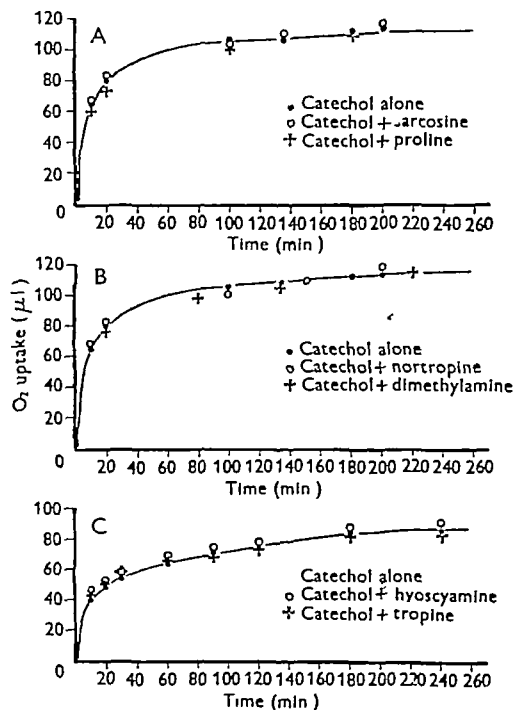


Fig 1 Oxygen consumed by belladonna polyphenolase catechol system, A and B with secondary amines and C with tertiary amines, all present in a concentration 10 times that of the catechol concentration (0.02 M).

The effect of addition of glycine to mixtures containing enzyme and equimolar concentrations of catechol and secondary amine

Reasons were given by James *et al* (1948) for supposing that the formation of a coloured complex made up of 1 mol of oxidized catechol and 1 mol of amino acid is an intermediate in the oxidation of amino acids by this enzyme system. Since secondary amines may be used as substitutes for amino acids in the formation of a coloured compound, the coloured compounds which they form might then act as intermediates in the oxidation of amino acids. The experiments which follow show that this is in fact true.

The results of James *et al* (1948) showed that even in the presence of a tenfold excess of secondary amine, no extra O_2 uptake occurred apart from that which was found with catechol only.

Manometric experiments were performed in which 0.05 M catechol (0.1 ml.) was added from a Kellin cup to the main chambers of manometer vessels containing 50 mg enzyme in 1.8 ml. buffer pH 7.8 and 0.05 M secondary amine (0.1 ml.) Glycine (0.5 ml, 0.1 M) was placed in the side

arms of some of the vessels, and it was thus possible to begin the oxidation of the catechol by dislodging the Keilin cups, and then to add the amino acid after any desired interval.

The oxidation of glycine in the presence of dimethylamine Fig 2 shows the results of experiments in which glycine was added after 20 min (A) and 75 min (B) to flasks containing (i) enzyme, catechol and dimethylamine (curves a and b), (ii) enzyme and catechol without dimethylamine (curves c and d) (curves e and f are controls to which no glycine was added)

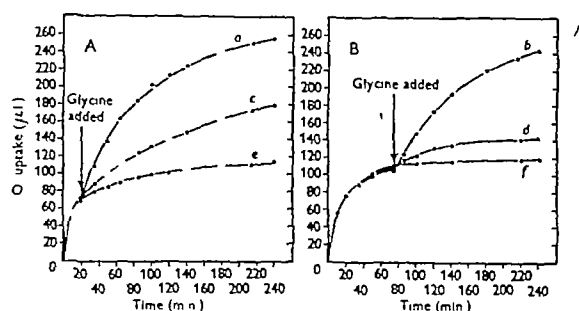


Fig 2 The effect on O₂ uptake of adding glycine after A, 20 min and B, 75 min to mixtures containing enzyme, catechol and dimethylamine (a and b), and enzyme and catechol without dimethylamine (c and d). Curves e and f are controls to which no glycine was added

Curves e and f show the O₂ uptakes of control flasks containing enzyme with catechol and dimethylamine to which no glycine was added, and in these the O₂ uptake stopped when 2 atoms O₂/mol catechol had been absorbed. The O₂ uptakes of systems a, c and e (Fig 2A) were almost indistinguishable from each other during the first 20 min, although purple colours had developed in a and e. At the end of this time, the glycine was tipped from the side arms of vessels a and c. A vigorous additional O₂ uptake then commenced in a, while in c also the glycine was oxidized, but at a noticeably slower rate. After 4 hr, i.e. 220 min after the addition of the glycine, the oxidation in the presence of dimethylamine had accounted for a total additional uptake of 145 μl O₂, while in its absence only 70 μl had been absorbed. In Fig 2B the O₂ uptakes of the three systems b, d and f were very similar until the glycine was tipped in after 75 min, and, by this time, the enzyme had almost completely oxidized the added catechol. In this case also, the addition of the glycine to the flask containing dimethylamine produced an immediate extra O₂ uptake (curve b) and 165 min later the total amount of O₂ uptake due to the added glycine was 130 μl. The contrast with curve d (no dimethylamine), was even more marked than after 20 min, since the addition of glycine produced only a small additional uptake of 25 μl.

The O₂ uptake curves in Fig 2, and a comparison of the amounts of glycine oxidation show clearly that glycine is oxidized much less readily when its addition, in the absence of dimethylamine, is delayed until after oxidation of catechol has begun, and, if this has progressed nearly to completion, the amount of glycine oxidation is very small indeed. When dimethylamine is present at the outset, there is no such diminution in the rates of oxidation when the addition of glycine is delayed to successively later stages. Indeed, when glycine was added after 75 min, the oxidation was slightly more rapid than that after 20 min, since almost equal amounts of glycine had been oxidized in the two cases at the end of 4 hr.

The oxidation of glycine in the presence of sarcosine and proline It was found that sarcosine and proline, which are not themselves oxidized under the experimental conditions, acted in the same way as dimethylamine. When glycine was added after 100 min

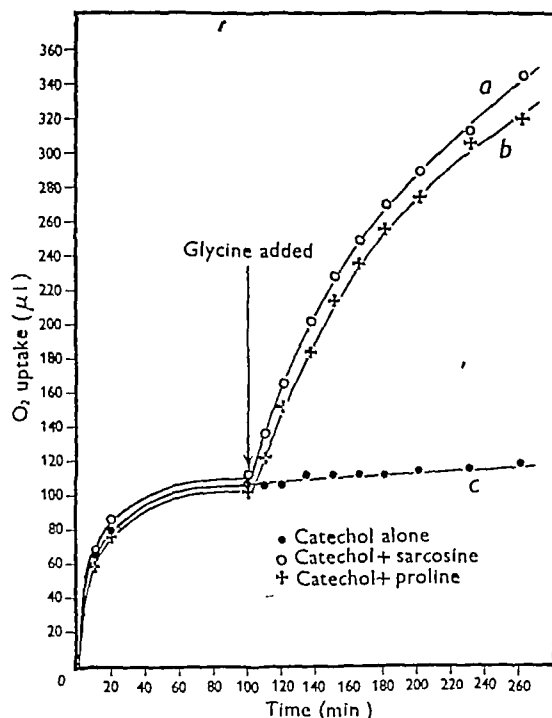


Fig 3 The effect on O₂ uptake of adding glycine after 100 min to (a) enzyme, catechol and sarcosine, and (b) enzyme, catechol and proline. Curve (c) is a control without added glycine

to flasks containing sarcosine or proline, in addition to catechol and enzyme (curves a and b in Fig 3), a rapid O₂ uptake began in each case. The oxidation of the catechol was almost complete when the glycine was added, and very little further O₂ uptake

The Coenzyme-like Action of Tryptophan in the Biosynthesis of Nicotinamide by *Bact coli*

By P ELLINGER and M M ABDEL KADER

2, 4, 5 and 7 methyl tryptophans (2mm) prepared and kindly supplied by Dr H N Rydon (1948) inhibit *in vitro* completely the synthesis of nicotinamide (Nam) in an ammonium lactate (am lact) medium containing ornithine (2mm) by *Bact coli* without affecting their growth. This inhibition, in combination with results by Heidelberger, Gullberg, Morgan & Lepkowsky (1948) that after intake of tryptophan- βC^{14} the eliminated Nam metabolites were not radioactive, suggested the possibility that tryptophan was not directly converted into Nam, but acted only as catalyst of the synthesis.

This hypothesis was tested by the following experiments.

Washed coli, broken up coli, saline washings of the latter, undialyzed and dialyzed, and the washed cell fragments were tested for their activity to synthesize Nam in saline phosphate or am lact

media containing no addition, tryptophan, ornithine or both of them in mM concentrations. While the washed cell fragments had lost all Nam synthesizing ability the four other preparations showed essentially identical behaviour.

No Nam was formed in saline phosphate medium in the absence or presence of tryptophan even when glucose was added as source of energy. In am lact or when ornithine was added to either medium Nam formation occurred which increased by three to seven times when tryptophan (1mm) was added to am lact. The already considerably greater Nam synthesis from ornithine was little increased in am lact and not in saline phosphate.

These results favour the conception of a coenzyme like action of tryptophan in the bacterial Nam synthesis.

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Investigations on Porphyrin Formation in Rabbits with the aid of ^{15}N By HELEN M MUIR and A NEUBERGER (National Institute for Medical Research, Hampstead, N W 3)

Shemin & Rittenberg (1946) have shown that the nitrogen of glycine is utilized specifically for the synthesis of porphyrins in the rat and in man. The present investigations were carried out in order to establish whether the glycine N enters into all four pyrrole rings. For this purpose it was necessary to elaborate micro methods for the degradations of porphyrins. Rabbits were fed 300 mg glycine (30 atom % excess ^{15}N) 1 kg body weight over 4 days and killed 2 weeks later. From the haemoglobins protoporphyrin methyl ester was obtained which was reduced with Pd and methyl methacrylate

polymer to mesoporphyrin ester (80 % yield). The latter was oxidized to methylmaleimide and haematinic acid in 65-70 % yield. These two substances were obtained crystalline. The ^{15}N contents of both substances were similar, suggesting that glycine or a metabolic derivative of glycine is the precursor of the nitrogen in both acidic and non-acidic pyrrolic rings. Experiments carried out on rats, in which the ^{15}N content of haemin was compared after feeding equivalent amounts of isotopic glycine and ethanalamine, indicated clearly that the latter is not a precursor of the porphyrin structure.

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The Micro-estimation of Citric Acid By H WEIL-MALHERBE (Runwell Hospital, nr Wickford, Essex)

The methods in which citric acid is estimated as pentabromoacetone consist of two phases (1) the conversion of citric acid into pentabromoacetone, (2) the estimation of pentabromoacetone formed. Most recent modifications concern the latter, whereas the former has hardly been changed since it was

described by Stahre in 1895. However, the use of KMnO_4 as oxidizing agent is not without danger as an excess must be avoided, furthermore, recoveries are often low in presence of other oxidizable substrates. In the modification proposed citric acid is oxidized at 50°C by vanadic acid in $10\text{N-H}_2\text{SO}_4$ in

followed manometrically, by CO_2 evolution. The breakdown was very sensitive to nicotinamide, 10^{-3} M-nicotinamide reduced the rate of breakdown of 3×10^{-4} M-cozymase to half its uninhibited value. The optimum pH of the reaction was c 7, it was

almost unaffected by many inorganic salts. The system responsible for the reaction remained associated with tissue debris in isotonic solutions, and was fairly stable at 0 or 37° but inactivated rapidly at 70° . It was easily obtained in solution in water.

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A Physical and Chemical Study of Bence-Jones Protein with especial reference to its Methionine Content and the possible significance of this By C. E. DENT and G. A. ROSE (*Medical Unit, University College Hospital, W.C. 1*)

The urinary protein passed by a patient with multiple myelomatosis has been studied. It gave the classical heat reactions for Bence-Jones protein (Osgood & Haskins, 1931). On electrophoretic analysis (by Dr E. L. Alling) it gave one slightly asymmetrical peak migrating with the mobility of fibrinogen. Salting-out experiments with sodium and zinc sulphates failed to reveal the presence of more than two constituents of which one comprised about 90%.

Analysis of the hydrolysate by paper chromatography (Consden, Gordon & Martin, 1944) revealed the presence of all the common amino acids except methionine and hydroxyproline. Methionine was later confirmed to be absent by rat growth experiments, by microbiological assay (by Dr J. G. Heathcote), by the colorimetric chemical method of McCarthy and Sullivan as modified by Horn, Jones & Blum (1946) and by a gravimetric chemical method (Lugg, 1938; Masters, 1939). Supplementation with methionine allowed the protein to support the growth of rats at the same rate as when they were fed casein.

It seems that the absence of methionine in this sample, if confirmed for other samples, could provide further evidence for the abnormal nature of Bence-Jones protein. Very few proteins are methionine free and all the main fractions of human plasma protein contain significant quantities (Brand, Kassell & Saidel, 1944).

Our patient excreted for some months before death about 36 g. daily of what therefore appears to be a fairly pure protein. Such large protein pro-

duction is not known to occur in normal or neoplastic tissues. The daily output was not related to his clinical condition or to the dietary or intravenous intake of protein, nor did it rise after feeding back by mouth 50 g. of his own Bence-Jones protein in addition to his normal diet. Other workers have failed to find Bence-Jones protein in normal body fluids (Kydd, 1934) or bone marrow (Martin, 1947). Our methionine analyses also suggest that it is probably foreign to the body.

It is suggested that the above facts relating to the protein could be explained if multiple myelomatosis were the result of an infection by a virus, which stimulated the plasma cells to reproduce rapidly, as in the case of the white cells in fowl leukaemia, and if Bence-Jones protein were the protein which, when attached to a nucleic acid, comprised the virus itself. There are analogies in the behaviour of known viruses which make this theory feasible. They may grow so rapidly as to comprise the major portion of an organism (Stanley, 1937), and may produce pure protein as well as nucleoprotein (Markham, Mathews & Smith, 1948). Further, virus proteins have been usually found to be methionine free (Block & Bolling, 1947, p. 304; Chandler, Gerrard, du Vigneaud & Stanley, 1947; Knight, 1947).

In the plasma cells of myeloma patients treated with stilbamidine, Snapper, Mirsky, Ris, Schneid & Rosenthal (1947) find basophil inclusion bodies which they attribute, on the basis of staining reactions, to the presence of a 'foreign nucleoprotein'. We believe this could be the virus in question.

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Utilization of Acetate by Tissues of the Ruminant By S J FOLLEY and T H FRENCH (National Institute for Research in Dairying, University of Reading)

Utilization of acetate by liver and kidney of small animals has been shown by various authors (see Bloch, 1947) Since ruminants absorb considerable quantities of lower fatty acids, mainly acetic, produced by fermentation of carbohydrates in the rumen (see Elsdon & Phillipson, 1948), the metabolism of acetate by ruminant tissues is of considerable interest

We have studied the respiratory metabolism of tissue slices (mainly lactating mammary gland) from ruminants, in presence of acetate, by the method of Dickens & Simer (1931) which enables net acid production or utilization to be conveniently measured Results so far indicate no unusual ability of ruminant liver (sheep, goat) to utilize acetate as compared with rat liver Kidney however shows a high Q_{O_2} with acetate (25-27), much greater than with glucose (17), and a marked acetate utilization ($-Q_{acid} = 8-11$) which exceeds that of rat kidney Lactating mammary gland from ruminants also exhibits striking utilization of acetate but with high R Q, in contrast to gland from the rat and mouse which does not appear to utilize acetate

With 0.02M acetate as substrate, results (each determination in duplicate) were obtained with lactating mammary gland as follows

Mean for six goats

$$Q_{O_2}, -6.2 \pm 0.5, \quad Q_{acid}, -4.2 \pm 0.7, \\ R Q, 1.20 \pm 0.08$$

Values for two cows

$$Q_{O_2}, -3.6, -7.3, \quad Q_{acid}, -1.6, -4.4, \\ R Q, 1.06, 1.15$$

Mean for six rats

$$Q_{O_2}, -4.8 \pm 0.3, \quad Q_{acid}, 0.6 \pm 0.2, \\ R Q, 0.75 \pm 0.06$$

By contrast, with glucose as substrate, which is utilized with high R Q by lactating mammary tissue from all non-ruminants (rat, mouse, guinea pig, rabbit) so far studied (Folley & French, 1948, and further unpublished work), ruminant udder tissue shows little if any increase in Q_{O_2} above endogenous values and the R Q is low

Mean values for ten goats

$$Q_{O_2}, -4.9, \pm 0.3, \quad Q_{acid}, 1.5, \pm 0.1, \\ R Q, 0.87 \pm 0.02$$

Values for two cows

$$Q_{O_2}, -2.2, -4.7, \quad Q_{acid}, 1.7, 2.3, \\ R Q, 0.64, 0.93$$

Under our conditions lactating mammary tissue of ruminants appears to differ from that of non-ruminants in its ability to metabolize acetate rather than glucose and the high R Q is consistent with the possibility that fat is synthesized from acetate These results may throw light on the origin of the short chain fatty acids (C_4-C_{14}) which are a feature of ruminant milk fat (Hilditch, 1947) If part of the milk fat in the ruminant is synthesized from acetate, a theory consistent with the demonstration that acetate can be built into fatty acid chains in the body (Rittenberg & Bloch, 1945) and with the high *in vivo* R Q of the ruminant udder (see Folley & French, 1948), the short chain fatty acids may well be intermediates of this process

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Nicotinic Acid in Cereals 2 The Effect on Chemical Assays of using Different Blanks

By CHLOE KLATZKIN, F W NORRIS and F WOKES

Klatzkin, Norris & Wokes (1948) found on germination of oats a significant increase in the nicotinic acid content, as determined by chemical and by microbiological methods. All the chemical results were, however, considerably higher than the microbiological results on the same samples. Further experiments on a new series of germinated oats samples have shown that this material contains large amounts of interfering substances (occurring especially in the husk) which are not completely removed in the adsorption step. Some of these develop interfering colours on addition of the amine reagent and are thus not allowed for in the solution blank (Melnick & Field, 1940, Dann & Handler, 1941) or total reagent blank (Bandier & Hald, 1939). The amine blank (Harris & Raymond, 1939) as employed on cereals by Kodicek (1940) makes better allowance for the interfering substances, but was found to give more variable results, perhaps because it does not allow for bleaching of the interfering colours by the cyanogen bromide. By employing a full series of blanks, more complete allowance can be made for these interfering substances and the chemical results brought nearer to the microbio-

logical results for both ungerminated and germinated oats and wheat, for both of which discrepancies had been found between chemical and microbiological results (James, Norris & Wokes, 1947). When using the full series of blanks such discrepancies still occurred with oats in the early stages of germination but were much reduced. On the other hand, with different samples of maize the discrepancies were scarcely altered. If the microbiological results had been lowered by the presence of an anti vitamin, this would be more likely to occur in oats or maize.

Chemical results as percentage of microbiological results

Cereal examined	Blanks used	
	Solution and total reagent	Full series
Oats, early stages of germination	164	131
Oats, late stages of germination	165	106
Wheat, early stages of germination	108	109
Wheat, late stages of germination	147	102
National flour	170	114
Maize (3 samples)	196	181

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Crystalline Retinene₂ By M K SALAH and R A MORTON (Biochemistry Department, University of Liverpool)

Retinene₂ from fresh water fish retinas (Wald, 1937) is a deeply yellow substance soluble in light petroleum and characterized by λ_{\max} 405m μ (CHCl₃) and 702–706m μ (SbCl₃ colour test).

Morton, Salah & Stubbs (1946) prepared retinene₂ from the unsaponifiable fraction of ling cod liver oil, which is rich in vitamin A₂, and similar preparations have been made from pike liver concentrates.

By feeding retinene₂ to rats (Morton, Salah & Stubbs, 1947), or by the use of aluminium isopropoxide (Ponndorf reaction) vitamin A₂ is produced. Retinene₂ (prepared by chromatography of mixtures containing much retinene₁), shows λ_{\max} 405m μ (CHCl₃) $E_{1\%}^{1\text{cm}}$ 1300 and in the colour test λ_{\max} 735m μ , drifting slowly to 705m μ (8 min).

0.24 g in 2 ml light petroleum, slowly cooled to

–70°, yielded a red oil from which the solvent was decanted. The oil was dissolved in fresh light petroleum and again cooled very slowly, yielding tiny orange crystals m.p. 57°, $E_{1\%}^{1\text{cm}}$ 385m μ 1320 (cyclohexane). Recrystallized from 1 ml of light petroleum the melting point rose to 59°, $E_{1\%}^{1\text{cm}}$ 1420. Further recrystallization raised the melting point to 61° and $E_{1\%}^{1\text{cm}}$ 385m μ , 1460 (cyclohexane) and subsequent recrystallization brought about no change.

The entire preparation, starting from 2 kg ling cod liver oil was repeated. The crystallized retinene₂ (prepared by P. D. Dalvi) was spectroscopically almost identical with the above product, but the melting point was 77°. Analysis agreed closely with C₂₀H₂₈O. The difference may be due to dimorphism or *cis trans* isomerism.

Properties of retinene₂

Solvent	λ_{\max} (m μ)	$E_{1\%}^{1\text{cm}}$	Inflexion (m μ)	Melting point (°)
cycloHexane	385	1460	c 310	61
Light petroleum	385	1460	c. 310	61
	390	1450	c 310	77
Chloroform	405	1420	c 315	61
	408	1357	c 320	77
Ethanol	390	1240	c 310	61
	400	1400	c. 315	77
SbCl ₃ reagent at first	735	4050	705	61
	735	3720	705	77
After 2½ hr	695	—	640, 580, 560, 510	61
	473	—	—	—
Sulphuric acid	735	—	At first 470 m μ later 695 and 505 appear on standing	—
Phosphoric acid	735	—	—	61
	700	—	—	77
Alcoholic <i>p</i> -aminobenzoic acid and conc HCl	545-560	—	Violet colour	—
Alcoholic aniline and conc HCl	525-535	—	Violet colour	—

The colour test, studied on the Beckman spectrophotometer shows that the 735 and 705 m μ maxima actually appear instantly at full strength, the latter, however, being an inflexion. Both bands

fade from the start, but the former fades much more quickly than the latter, so that after a few minutes an actual maxima at 705 m μ can be recorded.

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The Function of Carbonic Anhydrase in Gastric Mucosa By R. E. DAVIES and J. EDELMAN.
(Unit for Research in Cell Metabolism, Department of Biochemistry, The University, Sheffield 10)

Carbonic anhydrase was found in oxyntic cells by Davenport (1939, 1940a), who claimed to have inhibited carbonic anhydrase, and hence gastric HCl secretion, in dogs by thiocyanate (Davenport, 1940b). However, Feldberg, Keilin & Mann (1940) showed that acid secretion by cats was largely inhibited by thiocyanate when the gastric carbonic anhydrase, estimated at either 0 or 15° in extracts made by grinding the tissue with sand, was only 10% inhibited. No inhibition of acid secretion occurred when the gastric carbonic anhydrase, estimated as before, was between 80% and 'completely' inhibited by sulphanilamide (not less than 6.4×10^{-3} M in the blood) (Feldberg, Keilin & Mann, 1940, 1948). Davenport (1946) also failed to inhibit acid secretion in cats with 1.4×10^{-3} M thiophene 2-sulphonamide and withdrew the original claim. Similar results on turtles were reported by Anderson & Wilbur (1948).

Since the animals used were respiring normally, it seems certain that in all these experiments the

carbonic anhydrase of the blood and therefore of the stomach was not completely inhibited *in vivo*. However, less than 1% of the activity of the enzyme extractable from oxyntic cells is required to catalyze the uptake of CO₂ during the HCl secretion (Davies, 1948; Davies & Roughton, 1948).

A series of twenty-eight experiments has been carried out using acid-secreting frog and toad gastric mucosa (for technique see Davies, 1948). The addition of the carbonic anhydrase inhibitor *p*-toluenesulphonamide (Krebs, 1948) to final concentrations of 1.7×10^{-2} M always caused complete inhibition of acid secretion (3 expts), at 10^{-2} M the Q_{HCl} was lowered but acid secretion continued (4 expts) whilst 3×10^{-3} , 10^{-3} , 10^{-4} or 10^{-5} M solutions did not inhibit respiration or acid secretion (6 expts). Even 1.7×10^{-2} M *p*-toluenesulphonamide had no effect on the unstimulated respiration of frog liver, sartorius muscle, back skin, pancreas or gastric mucosa (10 expts) (compare Mann & Keilin, 1940). At this concentration the Q_{HCl} was inhibited slightly

during the first $\frac{1}{2}$ hr after addition and completely during the second $\frac{1}{2}$ hr and subsequently. There was an increase in the Q_{O_2} , this would be expected if the oxyntic cells were damaged (3 expts). Similar effects were obtained with 10^{-2} M-*p* sulphonamide benzoic acid and 10^{-3} M-prontosil soluble but not with 7×10^{-3} M sulphanilamide (6 expts) (Krebs, 1948, compare Feldberg *et al* 1940).

Carbonic anhydrase is needed to prevent accumulation of alkali in acid-secreting oxyntic cells (Davies & Roughton, 1948) and to allow the cell to transport Cl^- ions from the medium in exchange for HCO_3^- ions (compare Keilin & Mann, 1941). Complete inhibition of the enzyme should thus lead to the disorganization of acid-secreting oxyntic cells. The Q_{O_2} of acid-

secreting frog and toad gastric mucosa was reduced by 1.7×10^{-2} M-*p* toluenesulphonamide by the amount expected (usually 20–30%) if the respiration of the acid secreting oxyntic cells had been stopped (6 expts).

No trace of carbonic anhydrase activity was ever found in concentrated extracts of mucosa from such experiments, using the technique of Krebs & Roughton (1948). The extracted enzyme was inhibited more than 99%. No direct evidence is available as to the fraction of the enzyme inhibited in the intact oxyntic cells, but these experiments are in accordance with the theory advanced previously (Davies, 1948) that carbonic anhydrase plays a role in the process of acid secretion by these cells.

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A Study of the Partition Chromatography of Normal Urines By D. A. HALL (Department of Medicine, Leeds)

The use of partition chromatography for the analysis of gross changes occurring in urinary amino acid output in conditions such as acute yellow atrophy, and Fanconi syndrome, has been demonstrated by Dent (1947). In attempts to apply the method to an examination of the partial amino acid-urias observed in diabetes, it has been found that far less clear-cut results can be obtained. From a study of eighty cases of diabetes it appeared that only in forty cases was there any ninhydrin positive material present in detectable amounts, and only in twenty was marked amino-acid-uria existent. Hence it appeared desirable to determine whether normal urines showed similar variations, so that a basis for comparison could be obtained.

Normal urines from students and from hospital cases having no known metabolic disorder, have been examined, and the following facts, essential before comparison can be made with any but the more gross cases of amino acid-uria, have been observed.

There is no marked relationship between the total concentration of amino acids in the urine and the sum of the individual intensities of the separate spots on the chromatogram. The sum of the arbitrary values assigned to each spot, in the majority of

normals, lies below 5, whereas 25% of the diabetes samples have values in excess of 15. (On this scale the values for a urine sample from Fanconi's syndrome would have a value of about 50.) Variations in protein intake between 15 and 150 g. per day do not alter the amino acid output or the colour production to any corresponding degree, and although menstruation and pregnancy appear to alter the amino acid output slightly, the changes are not such as to raise the figures outside the normal range. There is, however, an indication that females excrete more amino-acids than males and that samples passed after long periods of retention in the bladder are likely to give deeper colours than those obtained under conditions of rapid diuresis.

The inferences to be drawn from these observations appear to be that if a sample, collected under normal conditions or under conditions affected merely by normal changes in internal environment, gives a total colour intensity, significantly above the value arbitrarily assigned the number 5, it may be assumed to be pathological. Also that, below this threshold value, considerable variations in normals occur, for the study of which the technique of paper chromatography is admirably suited.

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A Growth Factor for *Corynebacterium diphtheriae* from Yeast 1 Preparation By F W CHATTAWAY, DORIS E DOLBY, D A HALL and F C HAPPOLD (Departments of Biochemistry and Medicine, Leeds)

A concentrate has been prepared from an acid hydrolysate of yeast which has marked growth promoting activity for the Dundee (Barton) strain of *Corynebacterium diphtheriae*, on an otherwise characterized medium. The following flow sheet indicates the technique

Brewer's or Baker's Yeast
 ↓ 2N-HCl for 1½ hr
 Filtrate 1 + Residue 1
 ↓ PbO to pH 3
 Filtrate 2 + Residue 2
 ↓ 'Norite'
 Filtrate 3 + Residue 3
 ↓ Na₃PO₄ to pH 6.4
 Filtrate 4 + Residue 4
 ↓ Concentration at 40° C
 Filtrate 5 + Residue 5
 ↓ Phenyl hydrazine and NaAc in cold
 Filtrate 6 + Residue 6
 ↓ Alcohol to 50% with chilling
 Filtrate 7 + Residue 7

The material at the seventh stage of the purification had an activity, based on the dry weight of total solids, of 0.00001 µg/ml of medium. One third of the dry weight is reducing substance, but, although the majority of the activity is destroyed on the formation of an osazone, there is no direct evidence of the presence of a sugar in the active material. The activity is, however, also destroyed by ninhydrin, the material giving the characteristic peptide or amino acid colour, and by nitrous acid, and there is considerable evidence for the presence of peptides in the conjugate since the activity is partially destroyed on acid hydrolysis. In this, as in the absence of any simple method for the separation of the factor from the aqueous phase, and also in its presence in casein hydrolysate, there is a marked resemblance to streptogenin. The latter is, however, only 1/1000th part as active for this strain of *C. diphtheriae*.

Material of a similar if not identical nature has been shown to be present in liver extracts, both mammalian and from fish, and also, in human urine.

A Growth Factor for *Corynebacterium diphtheriae* from Yeast 2 Identification of the Components By F W CHATTAWAY, DORIS E DOLBY, D A HALL and F C HAPPOLD (Departments of Biochemistry and Medicine, Leeds)

Two dimensional partition chromatograms of a highly purified yeast fraction (Y 47) have shown the presence of twelve ninhydrin positive components. Of these, four have been identified by direct comparison with authentic specimens, as the free amino acids, glutamic acid, serine, glycine and isoleucine. Of the other eight, four at least are definitely peptide in nature, being hydrolyzed by acid. The other four are acid stable, but may still be peptide in nature. One of the acid labile peptides, P 2, present apparently to the extent of nearly one third of the α amino nitrogen of the preparation, on hydrolysis can be shown to be a tri-peptide containing serine, glycine and glutamic acid, partial degradation products, one of which has an *R_f* value comparable with that of seryl glycine, have also been obtained, and these appear to account for the presence of two of the other peptide spots. In view of this it is interesting to note that Woolley has claimed partial streptogenin activity for seryl glycol glutamic

acid and that P 2 is common to both Y 47 and streptogenin.

Treatment of the whole preparation with α-amino acid oxidase causes a decrease in activity, and the disappearance of those spots proved to be free amino acids and one of the acid stable components. Similar treatment after acid hydrolysis removes the rest of the activity, thus indicating that the activity may be assumed to be associated with at least two fractions, one of which is peptide in nature, the other apparently having the properties of a free amino acid. Activity has actually been shown to be associated with three of the components, two of the peptides, and a fraction which travels very slowly in both phenol and collidine and which is stable to acid hydrolysis.

All three fractions are required for complete growth, association of certain pairs, however, giving partial growth.

The Coenzyme-like Action of Tryptophan in the Biosynthesis of Nicotinamide by *Bact coli*

By P ELLINGER and M M ABDEL KADER

2, 4, 5 and 7-methyl tryptophans (2mm) prepared and kindly supplied by Dr H N Rydon (1948) inhibit *in vitro* completely the synthesis of nicotinamide (Nam) in an ammonium lactate (am lact) medium containing ornithine (2mm) by *Bact coli* without affecting their growth. This inhibition, in combination with results by Heidelberger, Gullberg, Morgan & Lepkowsky (1948) that after intake of tryptophan- βC^{14} the eliminated Nam metabolites were not radioactive, suggested the possibility that tryptophan was not directly converted into Nam, but acted only as catalyst of the synthesis.

This hypothesis was tested by the following experiments.

Washed coli, broken up coli, saline washings of the latter, undialyzed and dialyzed, and the washed cell fragments were tested for their activity to synthesize Nam in saline phosphate or am lact

media containing no addition, tryptophan, ornithine or both of them in mm concentrations. While the washed cell fragments had lost all Nam synthesizing ability the four other preparations showed essentially identical behaviour.

No Nam was formed in saline phosphate medium in the absence or presence of tryptophan even when glucose was added as source of energy. In am lact or when ornithine was added to either medium Nam formation occurred which increased by three to seven times when tryptophan (1mm) was added to am lact. The already considerably greater Nam synthesis from ornithine was little increased in am lact and not in saline phosphate.

These results favour the conception of a coenzyme-like action of tryptophan in the bacterial Nam synthesis.

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Investigations on Porphyrin Formation in Rabbits with the aid of ^{15}N By HELEN M MUIR and A NEUBERGER (National Institute for Medical Research, Hampstead, N W 3)

Shemin & Rittenberg (1946) have shown that the nitrogen of glycine is utilized specifically for the synthesis of porphyrins in the rat and in man. The present investigations were carried out in order to establish whether the glycine N enters into all four pyrrole rings. For this purpose it was necessary to elaborate micro methods for the degradations of porphyrins. Rabbits were fed 300 mg glycine (30 atom % excess ^{15}N) 1 kg body weight over 4 days and killed 2 weeks later. From the haemoglobins protoporphyrin methyl ester was obtained which was reduced with Pd and methyl methacrylate

polymer to mesoporphyrin ester (80% yield). The latter was oxidized to methylmaleimide and haematinic acid in 65-70% yield. These two substances were obtained crystalline. The ^{15}N contents of both substances were similar, suggesting that glycine or a metabolic derivative of glycine is the precursor of the nitrogen in both acidic and non acidic pyrrole rings. Experiments carried out on rats, in which the ^{15}N content of haemin was compared after feeding equivalent amounts of isotopic glycine and ethanalamine, indicated clearly that the latter is not a precursor of the porphyrin structure.

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The Micro-estimation of Citric Acid By H WEIL MALHERBE (Runwell Hospital, nr Wickford, Essex)

The methods in which citric acid is estimated as pentabromoacetone consist of two phases (1) the conversion of citric acid into pentabromoacetone, (2) the estimation of pentabromoacetone formed. Most recent modifications concern the latter, whereas the former has hardly been changed since it was

described by Stahre in 1895. However, the use of $KMnO_4$ as oxidizing agent is not without danger as an excess must be avoided, furthermore, recoveries are often low in presence of other oxidizable substrates. In the modification proposed citric acid is oxidized at 50° C by vanadic acid in 10N- H_2SO_4 in

occurred in the control system (curve c) which contained only catechol and enzyme, but in the presence of the secondary amines the oxidation of the glycine was particularly vigorous, and the curves of O_2 uptake were almost linear over 200 min

DISCUSSION

The results recorded by James *et al* (1948) showed that amino acids containing primary amino N react with an oxidation product of catechol produced by belladonna polyphenolase to form a compound with a deep red colour. Reasons were given for supposing the colour compound to have the structure of a *p* amino-*o* quinone. The results of this paper show that secondary amino N is also capable of forming strongly coloured, purple compounds. Tertiary amines, on the other hand, form no colour.

The most probable method of formation of the coloured compound is from *p* hydroxy-*o* benzoquinone (James *et al* 1948) (p. 635) by condensation of the amine and the hydroxyquinone at position 4. This condensation can readily be formulated for secondary as well as primary amines, but is impossible with tertiary amines. The results of the experiments with secondary and tertiary amines may, therefore, be regarded as providing confirmation of the suggested structure for the coloured compound.

Neither secondary nor tertiary amines are themselves oxidized in the system, the uptake of O_2 /mol of catechol remaining at 2 atoms. If the secondary amine united directly with the first oxidation product, *o* benzoquinone, to form the coloured compound, the O_2 consumption would be diminished to 1 atom. As it remains at 2 atoms, it seems safe to conclude that the quinone is first oxidized to the hydroxyquinone (consuming the second atom of O_2) as in the absence of the amine. This confirms the occurrence of the hydroxyquinone as an intermediate in the reaction chain of colour formation—

and hence of amino acid oxidation—as already suggested.

The coloured complex formed by secondary amines oxidized glycine as vigorously as an amino-acid coloured complex. The compounds derived from amino acids, which are secondary bases, and the complexes formed from secondary amines, which are tertiary bases, are relatively stable substances in dilute solution and do not rapidly condense, like the *p* hydroxy-*o* quinone, to non-reducible aggregates. This relative stability is no doubt the cause of their taking part in the oxidation of amino acids. This is clearly brought out by the results of Fig. 2 in which the addition of amino groups, after much of the *p* hydroxy-*o* quinone has had time to condense in their absence, brings about relatively little amino acid oxidation.

SUMMARY

1 Secondary amines react in the belladonna polyphenolase catechol system to produce compounds with a strong purple colour.

2 Oxygen uptake is not altered by the presence of the secondary amines.

3 Tertiary amines do not produce colours and are not oxidized.

4 The coloured complex formed by secondary amines oxidized glycine as vigorously as that formed by primary amino acids.

5 If the addition of the amine is delayed until the catechol oxidation product has had time to condense, oxidation of glycine is reduced.

6 The above facts are considered to confirm the *p* amino-*o* quinone structure of the coloured compounds formed with one molecule of amino-acid. Corresponding compounds with tertiary nitrogen are presumed to result from the condensation with secondary amines. The relative stability of these compounds enables them to participate in the oxidation of additional amino acid.

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